The Institute of Paper Chemistry

Appleton, Wisconsin

Doctor's Dissertation

Enzymatic Dimerization of Substituted Phenols

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June, 1961

ENZYMATIC DIMERIZATION OF SUBSTITUTED PHENOLS

A thesis submitted by

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for the degree of Doctor of Philosophy
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Appleton, Wisconsin

June, 1961

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GLOSSARY

1. Definition of Terms Used:

   Q_{O_2}-HQ, the oxygen quotient for an enzyme preparation (µl. of oxygen absorbed per hour per mg. of dry enzyme preparation) with hydroquinone (stabilized with sodium sulfite) as substrate.

   Q_{O_2}-CAT, as for Q_{O_2}-HQ but with catechol stabilized with sodium sulfite as substrate.

   HQU, hydroquinone units, the dry weight of enzyme preparation catalyzing the absorption of 10 µl. of oxygen per minute divided into the total dry weight of enzyme preparation available with hydroquinone (stabilized with sodium sulfite) as substrate.

   CATU, catechol units, as for HQU but with catechol stabilized with sodium sulfite as substrate.

2. Buffer Solutions:

   No. 1, 0.05M dibasic potassium hydrogen phosphate containing 5 grams per liter of ascorbic acid and saturated with n-butanol; pH 6.6 at 4°C.

   No. 2, 0.05M dibasic potassium hydrogen phosphate containing 5 grams per liter of ascorbic acid; pH 6.6 at 4°C.

   No. 3, 80 ml. of 1.0M sodium chloride, 20 ml. of 0.1M hydrochloric acid, and 40 ml. of 0.5M sodium veronal made up to one liter; ionic strength, 0.1; pH 9.0 at 1°C.

   No. 4, 0.1M potassium dihydrogen phosphate adjusted to pH 7.0 (25°C.) with 0.1M sodium hydroxide.

No. 5, 0.1M potassium hydrogen phthalate adjusted to pH 5.0 (25°C.) with 0.1M sodium hydroxide.

No. 6, 0.05M potassium dihydrogen phosphate adjusted to pH 7.0 (25°C.) with 0.05M sodium hydroxide.

3. Paper Chromatographic Developers:

XMF, xylene-methyl ethyl ketone-formamide (20:20:1).

EAPW, ethyl acetate-pyridine-water (8:2:1).

EAPWA, ethyl acetate-pyridine-water-acetic acid (5:5:3:1).

4. Qualitative Indicator Reagents for Substances on Paper Chromatograms:

DpNA, diazotized p-nitroaniline solution followed by an overspray of saturated aqueous sodium carbonate.

pA, p-anisidine hydrochloride dissolved in n-butanol.

DNPH, 12% aqueous hydrochloric acid saturated with 2,4-dinitrophenylhydrazine.

Wiesner reagent, 1% phloroglucinol in 12% aqueous hydrochloric acid.

Ninhydrin reagent, 0.2% 1,2,3-triketohydrindene in 95% ethanol.

5. Symbols:

A, area under a concentration gradient curve, sq. cm.

c, concentration, g./ml. of solution.

D, diffusion coefficient, sq. cm./sec.

D*, apparent diffusion coefficient, sq. cm./sec.

E, field strength, volts/cm.

(E_t), total enzyme concentration, moles/liter.

(ES), enzyme-substrate complex concentration, moles/liter.

$g(u)$, electrophoretic mobility distribution.

k_1, k_2, k_3, velocity constants.

K_m, Michaelis constant, moles/liter.

M, molecular weight, g./mole.

n, refractive index.

(P), product concentration, moles/liter.

R, gas constant, ergs/mole-°K.

s, sedimentation coefficient, 1×10^{-13} sec. (Svedberg unit).

(S), substrate concentration, moles/liter.

t, time.

T, absolute temperature, °K.

v, initial reaction velocity, moles/liter-minute.

V, maximum reaction velocity, moles/liter-minute.

$\bar{V}$, partial specific volume, g./ml.

$y_{max.}$, maximum ordinate of a concentration gradient curve, cm.

β, standard deviation of an electrophoretic mobility distribution or the heterogeneity coefficient.

ρ, solution density, g./ml.

ρ_o, solvent density, g./ml.

σ, second moment of a concentration gradient curve.

σ_o, second moment of a concentration gradient curve at zero time.

ω, angular rotation, radians/second.

INTRODUCTION

Lignin stands second only to cellulose as the chief organic raw material that is replenished annually. Since 1838, when Payen separated fibrous cellulose from its incrusting material, efforts have been made to understand lignin formation and its chemical composition. Analytical organic chemistry has met with limited success in isolating lignin fragments of dimeric or oligomeric nature having demonstrable relation to original lignin structure. Another approach to lignin chemistry has received wide interest in the past decade; that is, the enzymatic biosynthesis of phenylpropane polymers from coniferyl alcohol, a likely precursor of coniferous lignin.

The fact that mushroom extracts catalyze the polymerization of substituted phenolic compounds to form amorphous ligninlike products was first observed by Cousin and Herissey (1) in 1908. In 1933, Erdtman (2) studied the enzymatically catalyzed dehydrogenative polymerization of isoeugenol and suggested that lignin might be a dehydrogenation product of guaiacylpropane derivatives with an oxidized side chain. In 1943, Freudenberg and Richtzenhain (3) first observed the formation of a ligninlike precipitate in a dilute aqueous system of coniferyl alcohol and the juice squeezed from the cultivated mushroom (Psalliota campestris). A brief purification procedure for "mushroom enzymes" similar to the polyphenolase isolation of Keilen and Mann (4) was presented. Since that time, Freudenberg and co-workers (5-27) have investigated the enzymatic polymerization of p-hydroxycinnamyl (p-coumaryl); sinapyl; and, principally, coniferyl alcohols. The enzyme

preparations came from various sources, e.g., mushroom dehydrogenase (7-9), phenol dehydrogenase from mushrooms (10), mushroom press juice (8,9), phenol dehydrase (3,14), mushroom dehydrase (16,22), raw almond emulsin (8,9), mushroom redoxase (6,8,11,13,15,16), mushroom oxidoreductase (21), cambial oxidoreductase (21), horseradish peroxidase (12), mushroom laccase (12,27), and laccase (17,18,23-26). With the exception of mushroom laccase, little was published on the purification and characterization of these enzymes.

Emphasis was placed on the derivation and support of the hypothesis that the dehydrogenation polymer (DHP) of coniferyl alcohol and the secondary products preceding its formation *in vitro* are, respectively, the same as softwood lignin and the secondary products preceding its formation *in vivo* (24). This hypothesis is open to question and has not yet (1961) been unequivocally proven.

All secondary, products preceding DHP formation are optically inactive even though most of them contain one or more asymmetric carbon atoms. This fact is accounted for by the following theory propounded by Freudenberg, *et al.* (12,16,24-26). The removal of a hydrogen atom (proton and electron) from the phenolic hydroxyl of coniferyl alcohol is catalyzed by the enzyme. The free radical thus produced is believed to react without further enzymatic intervention. The free radical has several mesomeric forms which react with each other at random (see Fig. 1) to product dehydrodiconiferyl alcohol, dl-pinoresinol, or a quinone methide which can be stabilized by primary alcohols, carbohydrates, or water, to give a guaiacylglycerol derivative.

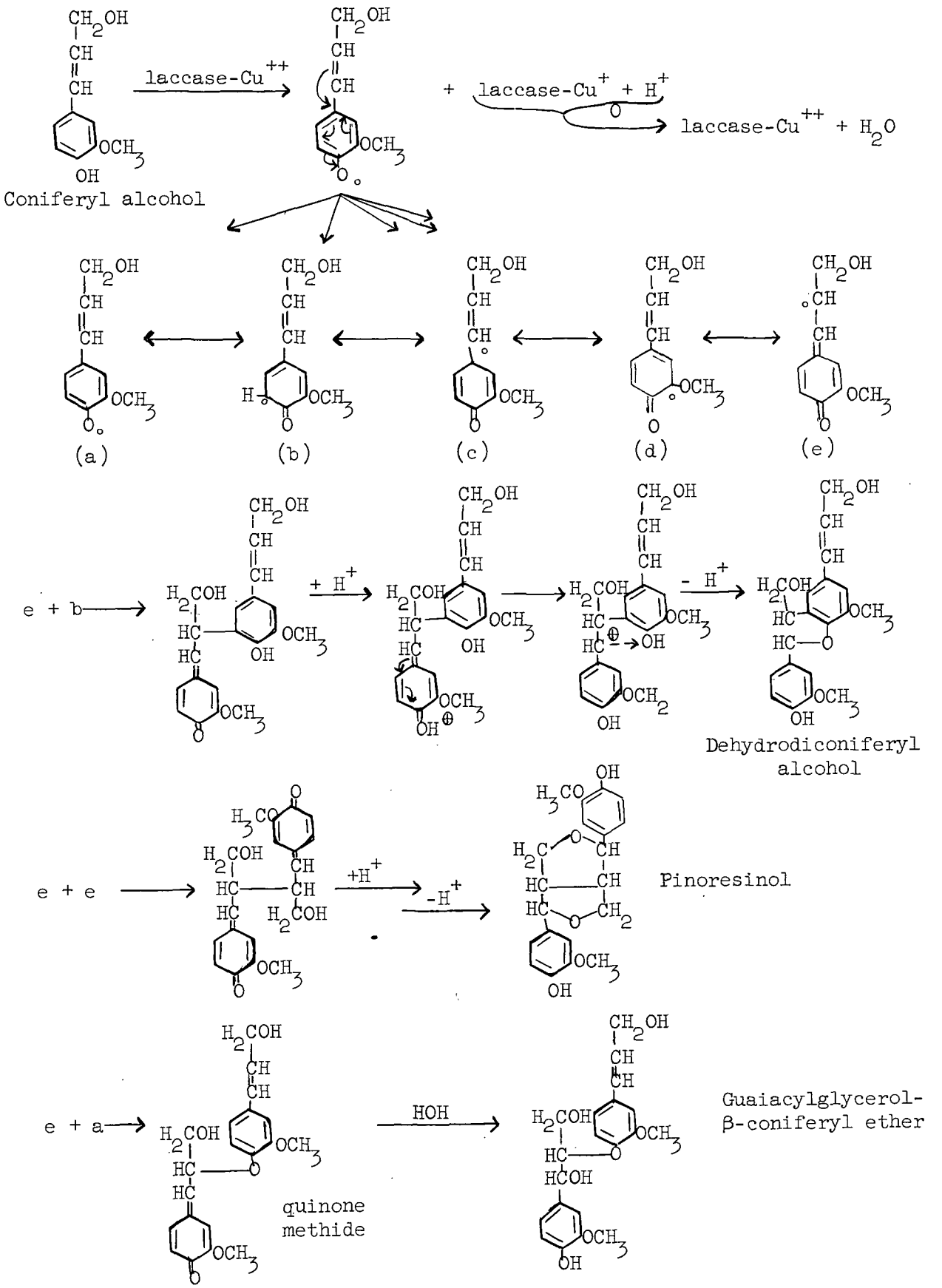

Figure 1. Reactions yielding principal dimers preceding DHP formation as hypothesized by Freudenberg, et al. (12,16,24,25) and Harkin (26)

Freudenberg, et al. (11,15-18) and others (28) have contributed greatly to the identification and proof of structure of eleven secondary products preceding DHP formation but no quantitative study of the disappearance of monomers or the rates of formation of the principal dimers has been made for these systems. A well-characterized laccaselike enzyme preparation from P. campestris has not been studied prior to this investigation. Several descriptive names for the enzyme catalysts have already been mentioned; those concerning mushrooms all describe the same kind of preparation, partially purified and most likely containing several different enzymes, one or more of which could be responsible for DHP formation.

Mason and Cronyn (29) presented evidence in 1955 which indicated that a polyphenoloxidase was not responsible for DHP formation. It was suggested that the active enzyme might be a laccase. In 1957, Higuchi (30) stated, "The 'phenol dehydrogenase' of Freudenberg is probably a laccase." In 1883, Yoshida (31) observed that the latex of the Japanese lacquer tree (Rhus vernicifera) contained a thermolabile nitrogenous substance that caused the hardening and darkening of the latex in air. Bertrand (32) isolated a similar enzyme from the Indo-Chinese lacquer tree (Rhus succedanea) and called it laccase in 1894. It was demonstrated that the polyphenolic compounds, urushiol and laccol, were enzymatically oxidized. The properties of laccase as well as several similar respiratory enzymes established prior to 1951 have been reviewed by Dawson and Tarpley (33), Lardy (34), and others (35-40).

In 1958, Freudenberg, et al. (12) completed moderate additional purification of the same mushroom extracts mentioned previously and

called the preparation mushroom laccase. Some of the properties of the mushroom laccase preparation were found to disagree with earlier data on laccase preparations (31-40). The aqueous mushroom laccase preparation was golden yellow with a blue fluorescence (26). Aqueous lacquer tree laccase is intensely blue (35,36). Although serving adequately for the in vitro polymerization of the secondary products preceding DHP formation, the mushroom laccase preparation of Freudenberg, et al. (12) was not characterized quantitatively.

In 1958, Fåhraeus, et al. (41,42) found that the mycelia of a white rot fungus (Polyporus versicolor Fries No. IIa) could be induced to form large amounts of an exoenzyme possessing laccaselike biological activity. Without the "inducer" (2,5-xylidene), the formation of laccase was negligible. After purification, the enzyme appeared homogeneous as judged by electrophoresis, ultracentrifugation, end-group determinations, and copper analyses. The pure preparation was deep blue in aqueous solution. The color was lost when the copper of the enzyme was in the reduced state. Also in 1958, Nakamura (43) purified the laccase of the Chinese lacquer tree (Rhus vernicifera) by column chromatography on an ion-exchange resin and by zone electrophoresis. The purity was confirmed by free electrophoresis and ultracentrifugation.

The purpose of this research was to provide a more complete characterization of the "mushroom laccase" enzyme. The quantitative determination of the rates of disappearance of DHP precursors, p-coumaryl, sinapyl, and coniferyl alcohols and the rates of formation of the principal dimers, dehydrodiconiferyl alcohol, dl-pinoresinol, and guaiacylglycerol-β-coniferyl ether in the coniferyl alcohol-enzyme system was made to gain an insight into the reaction mechanisms.

METHODS

MUSHROOM PROCUREMENT

Fresh young mushroom stem bases (Psalliota campestris) were obtained from the Wisconsin Mushroom Company of New Berlin, Wisconsin. Short storage prior to utilization was at 40°F. Tissue chemistry investigations of P. campestris (44,45) have shown that the enzyme possessing laccase-like biological activity is localized in the base of the mushroom stem, while tyrosinase is concentrated in the mushroom cap. The caps contain about a twentieth the laccaselike activity of the stems.

EXTRACTION PROCEDURES

The mushroom stem bases were extracted according to the schemes of Freudenberg, et al. (12) and Harkin (26) with two modifications. First, ascorbic acid was added to the buffer solution used for extraction to inhibit the formation of dark-colored melaninlike compounds (46). Second, the buffer solution used for extraction was saturated with n-butanol (47). This aided in breaking down permeability barriers in the fungal cell wall, thereby improving the yield of the cell-bound enzymes[a]. Detailed extraction procedures appear in Appendix I, page 95.

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[a]The purest preparation from this aqueous extract contained 3 electrophoretically distinct components (II, III, and IV, Fig. 4) and was designated pseudolaccase K. This author named the most active component, III, pseudolaccase.

FRACTIONATION PROCEDURES

The aqueous extract from the pressed mushroom stem bases was fractionated according to Freudenberg, et al. (12) and Harkin (26) with one modification; i.e., ascorbic acid was a part of buffer solution No. 2 for the reason cited earlier. Methanol fractionation separated enzymatic activities and concentrated them by precipitation. Detailed fractionation procedures appear in Appendix II; a flow diagram is included as a specific example.

DESALTING PROCEDURES

Gel filtration[a] (48,49) was used to desalt all buffered enzyme preparations in preference to dialysis. Two striking advantages supported the use of gel filtration: (1) Analytically complete desalting of a buffered enzyme preparation was accomplished in ten to sixty minutes,[b] and (2) a wide range of molecular size separations was possible.

Salt-free enzyme solutions were quick frozen in an acetone-dry ice bath in a manner yielding a high surface to volume ratio. Either immediately, or after storage in a freezer, the frozen enzyme preparations were lyophilized and subsequently stored at -20°C. over magnesium perchlorate in a desiccator.

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[a] Gel filtration refers to the use of Sephadex cross-linked dextran gels which are available through Pharacia Laboratories, Inc., 501 Fifth Avenue, New York 17, New York.

[b] The desalting time depends upon the sample and column size. Gel filtration is a new separation method similar to elution chromatography (50).

DETERMINATION OF ENZYMATIC ACTIVITY

Coniferyl alcohol was not suited to the measurement of enzymatic activity because it was not sufficiently soluble in water, the straight-line portion of the oxygen consumption versus time curve was too short, and the products of oxidation were insoluble. Many guaiacyl and syringyl compounds catalytically oxidized by pseudolaccase K possessed similar disadvantages. Hydroquinone does not have these disadvantages and most respiratory enzymes do not catalyze the oxidation of hydroquinone (33). Harkin (26) found that oxidases could oxidize hydroquinone when carriers were present; e.g., catechol. Most oxidases, except laccase and pseudo-laccase K, are inactivated by carbon monoxide. Therefore, pseudolaccase K activity was best determined by using hydroquinone as substrate under an atmosphere of 10% oxygen in carbon monoxide.

Specific activity, Q_{O_2}, was defined as the µl. of oxygen consumed per hour per mg. of dry enzyme preparation and an enzyme unit was defined as the amount of dry enzyme preparation that fixed 10 µl. of oxygen per minute. Both values depended on the conditions of measurement.

The procedure of Harkin (26) was used to determine enzymatic activity. Ten mg. of hydroquinone or catechol and 6 mg. of anhydrous sodium sulfite in 2 ml. of 0.05M phosphate buffer at pH 6.8 were placed in a Warburg flask with side arm. Five to ten micrograms of an enzyme preparation in 1 ml. of 0.05M phosphate buffer at pH 6.8 were placed in the side arm. After temperature equilibrium at 25°C., the contents of the side arm were dumped into the flask at zero time. Shaking rates greater than 110 cycles per minute did not increase the rate of oxidation. Absorption of carbon

dioxide by the buffer solution was undetectable at pH 6.8. The Warburg manometers were read every five minutes for one hour. All determinations were made in triplicate. The pressure readings were converted to volumes of oxygen absorbed with an IBM 610 computer program. Thermal barometer changes and manometer resettings were made through use of the computer program.

For miscellaneous techniques of activity measurement, such as glassware cleaning and Warburg apparatus care, see Umbreit, et al. (51).

ELECTROPHORESIS PROCEDURES

PAPER ELECTROPHORESIS

The Reco Model E-800-2 Electrophoresis Migration Chamber and Power Supply built by Research Equipment Corporation of Oakland, California was used for the qualitative separation of the pseudolaccase K and tyrosinase activities present in the partially purified enzyme preparation H_3-2. A study of the effect of pH on the rate of migration permitted the evaluation of the isoelectric points of the 3 components of pseudolaccase K. Details of the method appear in Appendix III, page 99.

FREE ELECTROPHORESIS

The Beckman/Spinco Model H Electrophoresis-Diffusion Instrument[a] was used for preparative and analytical electrophoresis and for the determination of diffusion and heterogeneity coefficients.

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[a]Beckman/Spinco Model H Electrophoresis-Diffusion Instrument Instruction Manual. Palo Alto, California, Beckman Instruments, Inc., 1958. 71 p.

Preparative Electrophoresis

Pseudolaccase H_3-2 was dissolved in 2 ml. of 0.1M buffer solution by storing overnight at 1°C. Immediately following centrifugation at 10,000 times gravity, the cold solution was introduced into the 2 ml. microcell. The microcell assembly has been described elsewhere (52). After 16 hours of countercurrent electrophoresis, the cell contents were cut into fractions as dictated by the schlieren optical system. The fractions were desalted, quick frozen, lyophilized, and checked for pseudolaccase and tyrosinase activities in the presence and absence of carbon monoxide. Undesirable enzymes were considered absent when the oxidation of hydroquinone and catechol was not impeded under an atmosphere of 90% carbon monoxide and 10% oxygen.

The results indicated that electrophoresis was a feasible means of further separating the components of enzyme preparation H_3-2. Large-scale preparative electrophoresis was carried out by zone electrophoresis techniques (53-55) in a cell which is described in Appendix IV, page 100, along with the details of operation.

Analytical Electrophoresis

Procedures used for preparative electrophoresis in the microcell were applicable to analytical electrophoresis. Solution concentrations were made an order of magnitude lower to permit proper light transmission through the solution leg of the microcell (0.5% as compared to 5.0%). Countercurrent operation was not necessary during analytical electrophoresis. Photographic records were taken during runs at pH 5, 7, and 9

on Eastman Kodak contrast process panchromatic film and were measured on the Wilder Microprojector adapted for the use of film and glass plates. Additional considerations are presented in Appendix V, page 103.

DETERMINATION OF HETEROGENEITY AND DIFFUSION COEFFICIENTS

The Beckman/Spinco Model H Electrophoresis-Diffusion Instrument equipped with schlieren optics permitted the measurement of diffusion coefficients with an accuracy of about 1%. The method was also sensitive in the determination of heterogeneity coefficients as developed by Alberty (56,57) and others (58-61). The experimental procedures were the same as those used for analytical electrophoresis. Theoretical details appear in Appendix VI, page 105.

ULTRACENTRIFUGATION

Two principal methods of ultracentrifuge operation exist in the largest sense: (1) Sedimentation velocity is used to study the translocation of molecules in solution under the influence of high centrifugal fields (up to 260,000 times gravity), and (2) sedimentation equilibrium is used to study the redistribution of molecules in a centrifuge cell at speeds low enough to permit the molecules to take up their equilibrium positions throughout the cell. The former has received wide use; the latter, limited use in the study of enzymes.

SEDIMENTATION VELOCITY

Solute molecules move under the influence of a high centrifugal field at a rate which is a function of their molecular weight and the

frictional resistance they experience in moving through the solvent. This method was, therefore, used to determine the sedimentation coefficient, s, and the purity of the solute. Sedimentation velocity data did not yield molecular weights directly but additional physical constants; i.e., diffusion coefficient and partial specific volume, were required, as seen in the Svedberg equation.

$$M = \frac{RTs}{D(1-\rho\bar{V})} \qquad (1)$$

For macromolecules of molecular weight below 250,000, the ultracentrifuge should be operated at top speed or a synthetic boundary cell used (52). Charge effects were assumed to have been damped out in buffer solutions of 0.1 ionic strength. The boundary was symmetrical or nearly so; thus, the movement of the maximum ordinate of the concentration gradient curve was a sufficiently accurate index of the movement of individual molecules. Goldberg (62) showed that it was actually the second moment of the concentration gradient curve which was a true measure of the sedimentation of solute molecules.

Sedimentation coefficients were conveniently evaluated by plotting the logarithm of the boundary position vs. time as indicated in Equation (2).

$$s = \frac{1}{\omega^2 x} \frac{dx}{dt} \qquad (2)$$

The distance between the maximum ordinate of the boundary and the axis of rotation was x.

SEDIMENTATION EQUILIBRIUM

Measurements of the concentration distribution at sedimentation equilibrium give, directly, the molecular weight of the sedimenting macromolecules. Both sedimentation velocity and sedimentation equilibrium require knowledge of the partial specific volume of the macromolecule and this will be discussed below. Despite this obvious advantage and the fact that the theoretical foundations for the equilibrium method are firmer than those for the sedimentation velocity method, the method of sedimentation equilibrium is rarely used to study enzymes. Detailed experimental operating procedures are available in the literature (52). For a homogeneous preparation at equilibrium conditions, the slope of the straight line on a plot of log $(1/\underline{x})(\underline{dc}/\underline{dx})$ vs. $\underline{x}^2$ multiplied by $4.6\underline{RT}/(1-\rho\,\overline{\underline{V}})\omega^2$ gives the molecular weight directly.

PARTIAL SPECIFIC VOLUME

The use of either sedimentation velocity or equilibrium ultracentrifuge data requires evaluation of the partial specific volume, $\overline{\underline{V}}$. For proteins, $\overline{\underline{V}}$ generally ranges from 0.70 to 0.75 ml./g. and the density, ρ, of the solution studied is normally close to unity. Hence, errors in the determination of $\overline{\underline{V}}$ in the term, $(1-\rho\overline{\underline{V}})$, are essentially multiplied by a factor of three in the calculation of molecular weight. Accurate values of $\overline{\underline{V}}$ are imperative.

Several methods are available for measuring partial specific volume (52). A density gradient column gave good precision with very small volumes of solution. Exact details of the construction and use of

bromobenzene-liquid paraffin columns appear in the literature (52,63). The apparatus used in this research is shown in Fig. 2.

REACTION RATE STUDIES

OXYGEN ABSORPTION METHOD

The simplest means of obtaining initial reaction rate data utilizes the Warburg constant volume respirometer based on the following stoichiometric reaction:

$$4 \text{ substrate molecules} + O_2 \longrightarrow 2 \text{ dimers} + 2H_2O \quad (3)$$

Coniferyl, sinapyl, and p-coumaryl alcohol concentrations from 0.0167 to 0.00011M were employed in the same manner as was hydroquinone for activity measurement, but the sodium sulfite was omitted (see page 8). Oxygen consumption was determined at pH 6.2, 6.7, 7.2, and 7.7 at 25°C. in 0.05M phosphate buffer solutions. This series of pHs included most physiological cases and the pH of maximum laccase activity on most substrates (33). Warburg manometers were read every five minutes for twenty to thirty minutes which permitted initial reaction rates to be accurately estimated. A least-squares analysis of a Lineweaver-Burk (64) plot of the reciprocal of the initial reaction rate, $1/v$, vs. the reciprocal of the substrate concentration, $1/S$, yielded the Michaelis constant, K_m, and maximum velocity constant, V (65). See Appendix VII, page 109.

RADIOISOTOPIC TRACER METHOD

An independent method for obtaining initial reaction rates for the coniferyl alcohol-pseudolaccase K system employed C^{14}-labeled coniferyl

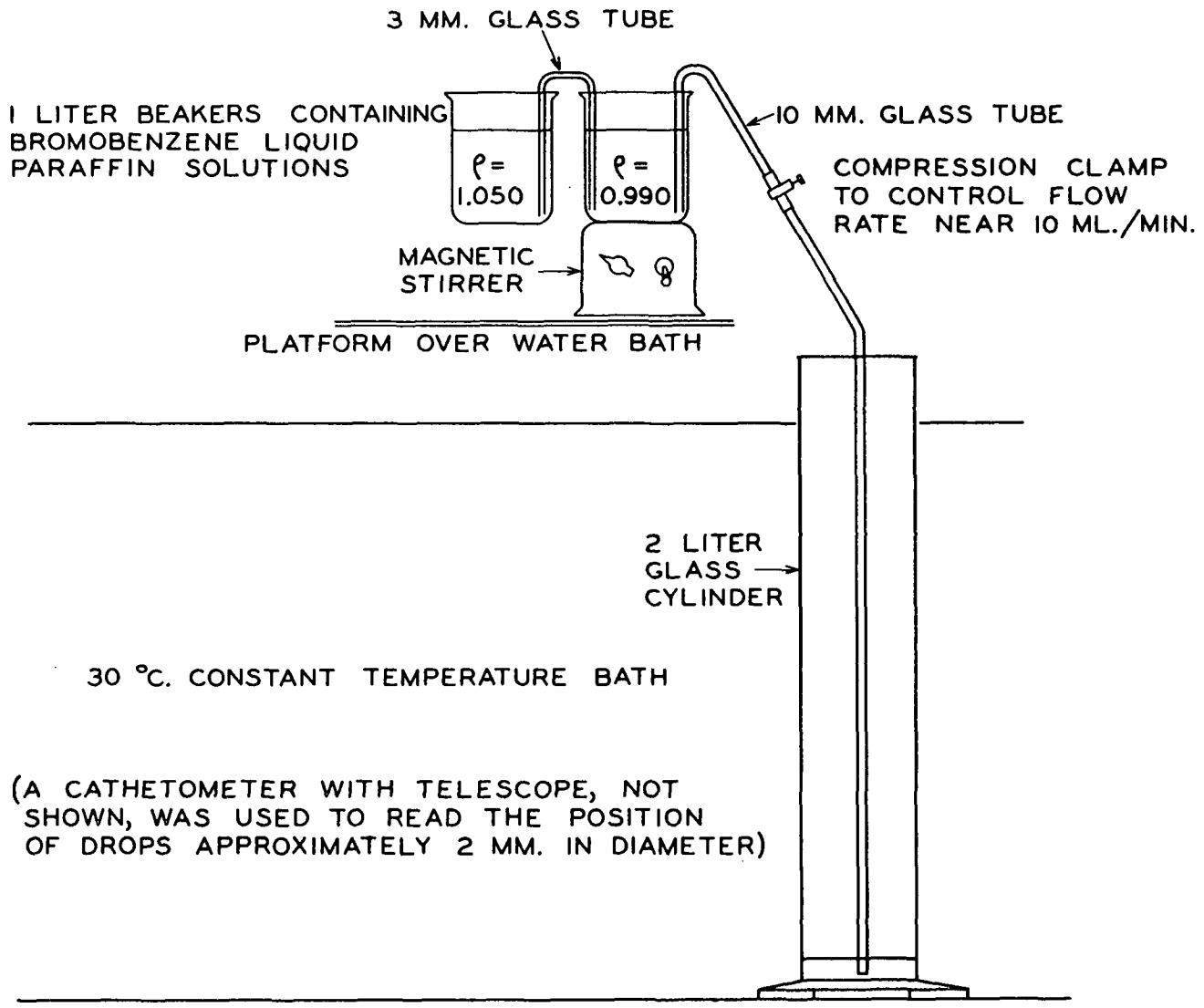

Figure 2. Density Gradient Column Preparation

alcohol. The experiments were also carried out at 25°C. in 0.05M phosphate buffer solution using the four pHs mentioned above. Concentrations of coniferyl alcohol ranged from 0.00572 to 0.000054M. The experiments with β-C^{14}-coniferyl alcohol were not run in triplicate as were those for obtaining oxygen absorption data; however, the abundance of data points gave excellent precision on a Lineweaver-Burk plot.

Three ml. of coniferyl alcohol solution were aerated while temperature equilibrium was attained. At zero time, 1 ml. of enzyme solution was added, with stirring, to the aerated coniferyl alcohol solution. At 2, 5, 10, and 15 minutes, 1 ml. of the reacting system was added to 0.5 ml. of 0.02M sodium cyanide solution to stop the reaction by inactivating the enzyme. Aliquots of the inactivated system were spotted on Whatman No. 1 chromatography paper previously steeped in formamide-ethyl acetate (1:5) and air dried. The chromatograms were developed with xylene-methyl ethyl ketone-formamide (20:20:1) (XMF) for approximately eight hours (20,26). The chromatograms were sprayed with diazotized p-nitroaniline (DpNA) solution and an overspray of saturated sodium carbonate solution. The desired materials, now with characteristic color and R_{ca}[a], were cut from the paper and subjected to wet combustion as described by Van Slyke, et al. (66,67). In most cases the chromatographic developer (XMF) limited the spots of radioactive material to a size convenient for one wet combustion experiment. When necessary, two combustions were performed and the results added. The paper carrying

[a] R_{ca}, the displacement from the origin of a material on a paper chromatogram relative to the displacement of the respective substrate alcohol.

the radioactive material served as a source of carbon dioxide to permit efficient sweeping of the radioactive carbon dioxide into the Bernstein-Ballentine proportional counting tubes (68).

Manometric Determination of Carbon and its Radioactivity

The difficulties of low efficiency counting in the solid state were avoided by the convenient determination of the radioactivity of the unreacted coniferyl alcohol and principle dimers in the gaseous phase using the Bernstein-Ballentine (68) proportional counting system. The compound of interest and the chromatographic paper carrying it were converted to gaseous products by the wet combustion technique of Van Slyke and Folch (66) as modified for use with radioactive carbon samples by Van Slyke, Steele, and Plazin (67). The method involved complete chemical oxidation of the sample to carbon dioxide, subjecting the gases to alkaline hydrazine solution, separating the carbon dioxide from the other liberated gases, collecting the carbon dioxide in a proportional counting tube, bringing the gases within the tube to atmospheric pressure with methane, and counting the radioactivity present.

Calibration of Proportional Counting Tubes

Two characteristics of a proportional counting tube must be known. These are the nature of its counting plateau, and the effect of mixtures of radioactive and nonradioactive carbon dioxide on the counting efficiency.

The counting plateau of a proportional counting tube was that portion of the curve of counting rate vs. voltage which showed a minimum slope over

a range of voltages. In that region, small changes in applied voltage had little effect on the counting rate. With mixtures of C^{14}- and C^{12}-carbon dioxide and methane in a proportional counting tube, Van Slyke, et al. (67) demonstrated that the counting rate depended to a certain extent on the partial pressure of the total carbon dioxide present. The efficiency of counting in 100-ml. tubes was constant up to a carbon dioxide partial pressure of about 120 mm. of mercury, or ca. 7 mg. of carbon beyond which it gradually decreased. Details of the calibration experiments are given in Appendix VIII, page 112.

SYNTHESIS OF SUBSTRATE ALCOHOLS

REAGENTS

Technical-grade malonic acid (Eastman yellow label; m.p. 132 to 134.5°C.) was fractionally crystallized from hot water. The second fraction was large enough to furnish a uniform supply for all syntheses (m.p. 133.5 to 134.5°C.). (All melting points were uncorrected.) p-Hydroxybenzaldehyde and vanillin were Eastman white label, melting at 116 to 117°C. and 82 to 83.5°C., respectively. Syringaldehyde melting at 109 to 110°C. was furnished by Dr. I. A. Pearl. All additional reagents were used straight from the packaged container, except pyridine which was dried over potassium hydroxide.

REACTIONS

The Vorsatz adaptation of the Doebner modification of the Perkin reaction (69) was used to synthesize p-coumaric, ferulic, and sinapic acids in 500-mg. quantities. The reaction was carried out at 60°C. for

20 hours. Standard procedures (69) used malonic acid 100% or more in excess of the aromatic aldehyde with which it was to be condensed, but this was not feasible when radioactive malonic acid was to be employed. The ratio of aldehyde to malonic acid of 1:1 was found to be most satisfactory. An excess of aldehyde gave poorer yields and crude products of lower melting points.

The acids were esterified in 25 ml. of absolute ethanol containing 0.5 ml. of concentrated sulfuric acid as catalyst. A boiling chip was placed in the flask. After refluxing for 6 hours and cooling overnight, the solution was passed through a regenerated and washed Duolite A-2 resin column (1 x 50 cm.) in which the water was replaced with absolute ethanol (70). The flow rate through the column was 2 ml. per minute. The solution and washings from the column were combined and reduced to dryness at room temperature on a rotating evaporator.

The dry ethyl esters, whether crystalline or sirup, were dissolved in 2 ml. of acetic anhydride with 2 ml. of dry pyridine as catalyst. The reaction was carried out over 16 hours at 60°C. The products were poured into 3 ml. of concentrated hydrochloric acid and 10 g. of crushed ice and left overnight before filtering, washing, and drying. The once-recrystallized acetylated ethyl esters were used for reduction to the respective alcohols. p-Coumaryl alcohol was prepared according to Freudenberg and Gehrke (7) except that the apparatus in Fig. 3 was used. Coniferyl alcohol was prepared according to Freudenberg and Hubner (15) also using the apparatus in Fig. 3. Dr. I. A. Pearl supplied sinapyl alcohol after several attempts to prepare crystals failed.

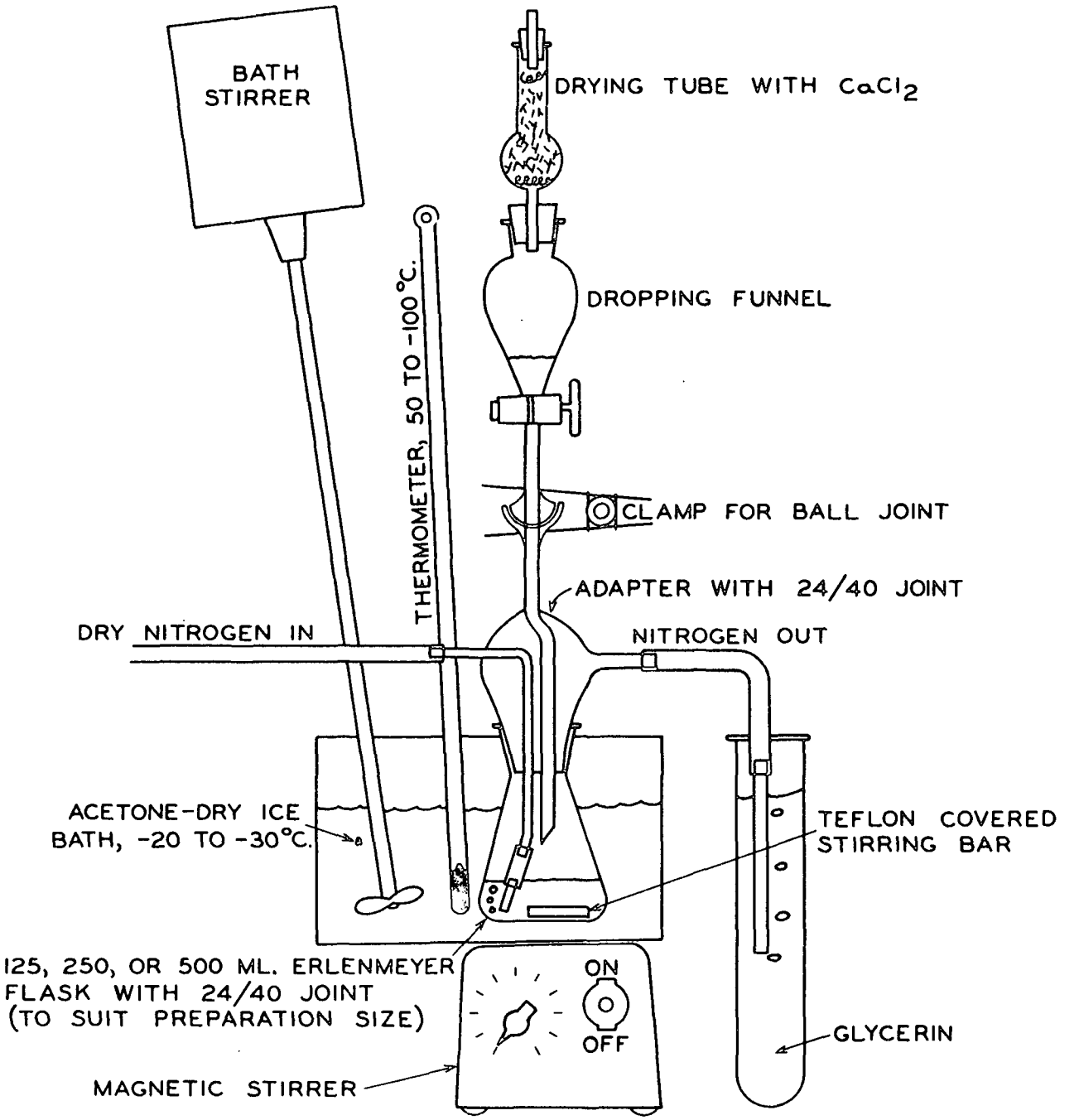

Figure 3. Apparatus for Reducing Acetylated Ethyl Esters with Lithium Aluminum Hydride in Dry Ether Solution Under Nitrogen

HYDROLYSIS OF PSEUDOLACCASE K

In order to analyze the carbohydrate portion of the enzyme, 50 λ of pseudolaccase K were hydrolyzed by adding 35 λ of 2N sulfuric acid and making the volume up to 100 λ. The acid solution was placed on a steam bath for 3 hours. The volume was maintained by occasional addition of water. The acid was neutralized by adding 3 mg. of solid barium carbonate and 10 λ of acetic acid to the solution. The barium sulfate was centrifuged down and the supernatant liquid spotted directly on Whatman No. 1 chromatography paper.

COPPER ANALYSIS

The amount of copper present in pseudolaccase K fractions was determined by first destroying the organic portion of the sample with concentrated nitric and perchloric acids and subsequently developing color with 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (bathocuproine) in n-hexanol as described by Borchardt and Butler (71). Bathocuproine in n-hexanol specifically complexes with cuprous ions to give an orange-brown solution which was measured colorimetrically at 479 mμ.

NITROGEN ANALYSIS

Accurately weighed 2-mg. samples were subjected to pyrolytic oxidation in the presence of Cuprox (cupric oxide). The nitrogen was swept through a Coleman Nitrogen Analyzer[a] by carbon dioxide, the latter was absorbed in potassium hydroxide, and the nitrogen was measured in the Dumas manner.

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[a]Coleman Nitrogen Analyzer, Maywood, Illinois, Coleman Instruments, Inc., 1959.

SPECTRA DETERMINATIONS

The ultraviolet and visible spectra determinations for pseudo-laccase K in phosphate buffer solution (No. 4) were made by Mr. Lowell Sell with a Beckman Model DK-2 ratio recording spectrophotometer.

AUTHENTIC SAMPLES OF COMPOUNDS

Authentic samples of dehydrodiconiferyl alcohol, dl-pinoresinol, dl-syringaresinol, and a mixture of the secondary products preceding DHP formation (20) were supplied by Professor Karl Freudenberg of Heidelberg University, Heidelberg, Germany. An exemplary chromatogram of the mixture developed in XMF was also obtained. Authentic sinapyl alcohol was supplied by Dr. I. A. Pearl.

RESULTS

EXTRACTION AND FRACTIONATION

The ascorbic acid in buffer solutions No. 1 and 2 completely inhibited the formation of dark-colored melaninlike materials. The n-butanol improved the yield of enzymatically active components by 100% when compared with preliminary extractions employing buffer solutions devoid of n-butanol. The increase of specific activity up to and including the desalted crude preparation H_3-2 is summarized in Table I.

TABLE I

SPECIFIC ACTIVITIES AND YIELDS DURING FRACTIONATION

Preparation	Dry Weight, g.	Q_{O_2}-HQ	Q_{O_2}-CAT	HQU	CATU
A_3[a]	482.	43	94	34,700	75,200
D_3[b]	37.2	194	228	12,000	14,150
G_3-2[c]	3.52	457	504	3,410	3,765
H_3-2,1[d]	0.348	2,377	2,515	1,380	1,462
2	0.741	2,871	3,208	3,545	3,950
3	0.196	1,284	1,878	420	611
4	0.117	379	486	74	95
5	0.241	43	58	17	23
6	0.706	--	--	--	--
7	1.060	--	--	--	--
H_3-2, Total	3.409			5,436	6,141

[a]Aqueous mushroom stem base extracts (see Appendix I).

[b]One methanol fractionation (see Appendix II).

[c]Two methanol fractionations (see Appendix II).

[d]Seven fractions of H_3-2 were collected while desalting G_3-2 with Sephadex G-25.

Fractions H_3-2,1 and 2 were combined to yield crude pseudolaccase H_3-2 for further purification. Fractions H_3-2,3 through 7 were not used. The total units of enzyme increased after desalting with Sephadex G-25 because low molecular weight materials which presumably inhibit enzymatic activity were removed. These inhibiting materials, below about 3000 molecular weight, possessed a golden-yellow color.

The crude pseudolaccase H_3-2 possessed 5 distinct components; all 5 exhibited enzymatic activity. Figure 4 is the schlieren electrophoresis pattern for 1% H_3-2 in phosphate buffer solution (No. 4) after 9 hours of electrophoresis at 8 ma. in the 11 ml. Tiselius-type electrophoresis cell.

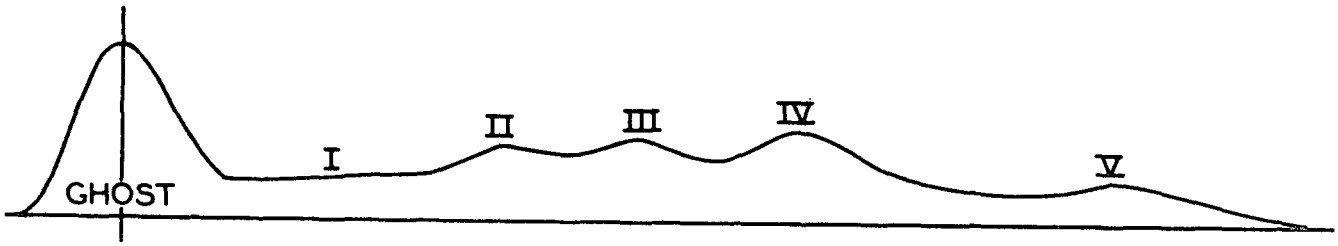

Figure 4. Schlieren Electrophoresis Pattern of Pseudolaccase H_3-2
(See the Text for the Experimental Conditions)

Component I was identified as tyrosinase; it was inactivated by carbon monoxide; it catalyzed the oxidation of catechol, p-cresol, and tyrosine; and it was inactive on hydroquinone, p-phenylenediamine, and guaiacyl and syringyl compounds. Component V vigorously catalyzed the oxidation of p-phenylenediamine, but not catechol, and was inactivated by carbon monoxide. Components II, III, and IV were present in pseudolaccase K and are completely discussed later. They were not inactivated

by carbon monoxide; they catalyzed the oxidation of most guaiacyl and syringyl compounds, hydroquinone, catechol, and p-cresol; and they were inactive on tyrosine and vanillin.

ISOELECTRIC pH OF PSEUDOLACCASE K

Filter paper-stabilized ionograms were used to determine the rate of anodic migration of pseudolaccase K and tyrosinase as a function of pH at constant field strength. Both tyrosinase and pseudolaccase K activities produced brown colorations at the regions of highest concentration when catechol was used as chromophoric substrate in agar gel (see Appendix III). For both activities, the brown spots were surrounded by yellow halos above pH 7 and light green halos below pH 7. The tyrosinase activity was inhibited below pH 5.4 and above pH 8.

When p-phenylenediamine was used as substrate, only one intensely colored spot occurred. Its mobility was comparable with that of the faster brown spot when catechol was used as substrate. The spot was dark blue below pH 7, violet above pH 7, and surrounded by a halo of lesser intensity but the same color. The activity of pseudolaccase K was good from pH 3.6 to 9.0. At pHs greater than 9, the pseudolaccase K activity was inhibited. Figure 5 summarizes the data. After statistical analysis, the data yielded an isoelectric point between pH 3.47 and 3.62 for pseudolaccase K. The data for tyrosinase were more scattered, but upon extrapolation an isoelectric point one to two pH units below that of pseudolaccase K was suggested.

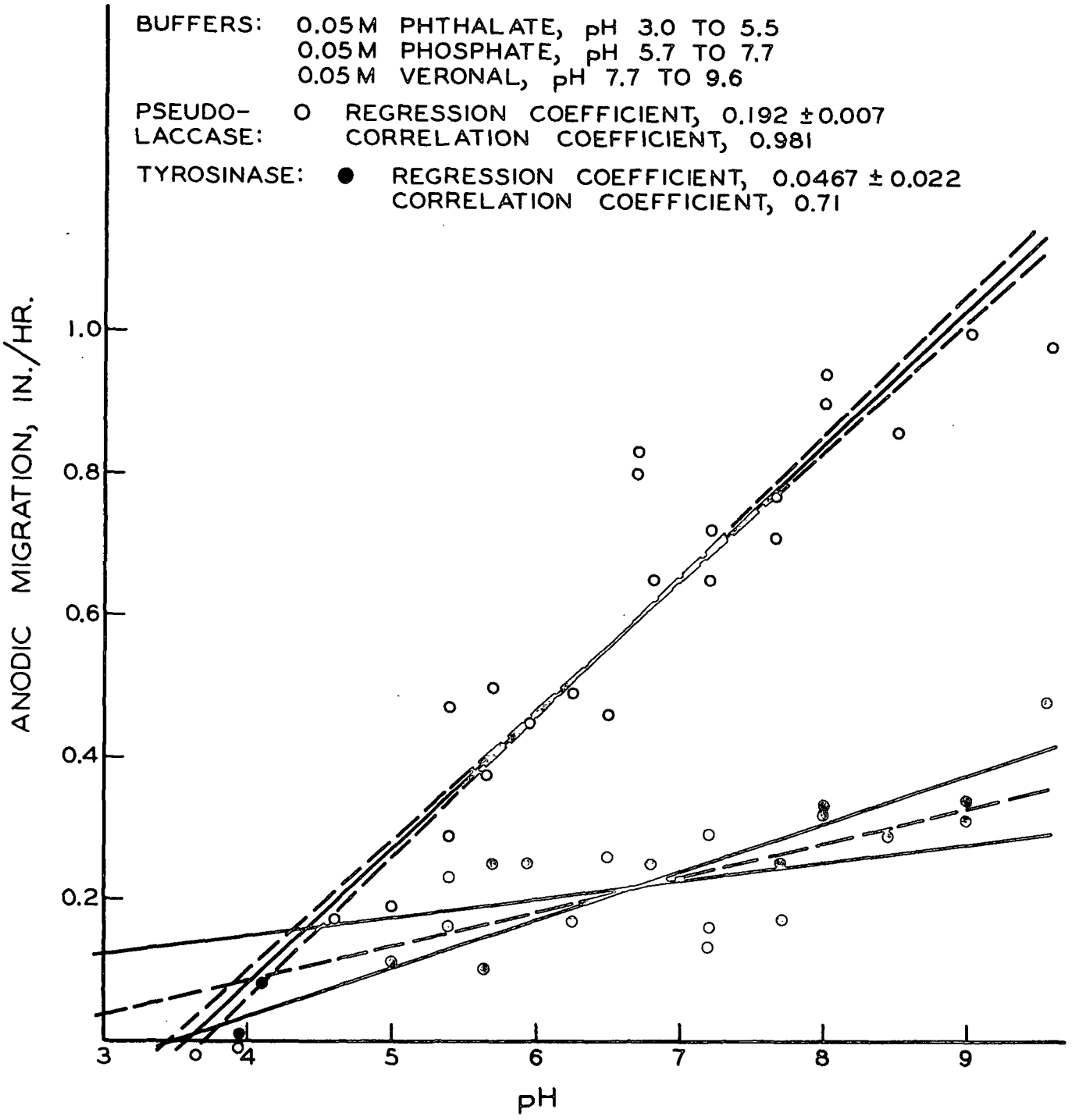

Figure 5. The Anodic Migration of Pseudolaccase K and Tyrosinase During Paper Electrophoresis at 400 Volts

ELECTROPHORESIS

PREPARATIVE ELECTROPHORESIS

Repeated countercurrent electrophoresis in the microcell indicated that electrophoresis as a preparative technique was capable of improving the specific activity of pseudolaccase K 12-fold. Enzymes inactivated by carbon monoxide were also removed from crude preparation H_3-2. Table II summarizes the results at various levels of pseudolaccase K purification on a specific activity basis.

Complete separation of pseudolaccase K and tyrosinase activities was not possible by a single zone electrophoresis experiment as Fig. 6 indicates. The cell described on page 100 was used. Veronal and phosphate buffer solutions (Nos. 3 and 4, respectively) were used at a current of 30 ma. for 30 hours. The fraction volume was ca. 4 ml. Catechol activity (o) was measured as described on page 102. The (+) and (-) at the bottom of the figure indicate the position of the anode and cathode, respectively. Fractions were combined as the idealized broken lines indicate. The fractions of a particular experiment were combined, after catechol activity analysis, to yield a pseudolaccase-rich fraction, a tyrosinase-rich fraction, and a fraction possessing a mixture of activities. Sufficient amounts of pseudolaccase-rich preparations were obtained, combined, and subjected to successive zone electrophoresis runs to achieve a complete separation of pseudolaccase K and tyrosinase after 3 successive experiments. See Fig. 7 which was run under the same conditions as described for Fig. 6. See also Table II. Table III summarizes the pertinent zone electrophoresis experiments of this research.

TABLE II

CARBON MONOXIDE SENSITIVITY OF ENZYME PREPARATIONS[a]

Preparation	Air		$CO:O_2::90:10$	
	Q_{O_2}-HQ	Q_{O_2}-CAT	Q_{O_2}-HQ	Q_{O_2}-CAT
J[b]	29,500	33,400	19,300	25,700
J_4[c]	34,500	34,500	34,500	34,500
Run 8[d]	33,200	36,200	33,200	33,200
Run 8[e]	30,000	37.800	30,000	29,200
Run 9[d]	32,700	35,600	32,700	32,700
Run 9[e]	32,300	36,500	32,700	32,700
Run 14[f]	33,600	34,600	33,600	33,600
Run 16[g]	35,300	35,300	35,300	35,300

[a]All values are the average of three determinations according to the procedure on page 8.

[b]Preparation J was the result of subjection of H_3-2 to countercurrent electrophoresis once.

[c]Preparation J_4 was the result of 4 successive countercurrent electrophoresis runs on H_3-2.

[d]Runs 8 and 9 were the result of subjection of H_3-2 to zone electrophoresis once; this fraction was rich in pseudolaccase activity (see Table III).

[e]As for [d] except this fraction was a mixture of pseudolaccase and tyrosinase activities (see Table III, page 30).

[f]Run 14 was the result of 2 successive zone electrophoresis runs on H_3-2; this fraction was rich in pseudolaccase activity (see Table III).

[g]Run 16 was the result of 3 successive zone electrophoresis runs on H_3-2; this active fraction was called pseudolaccase K (see Table III).

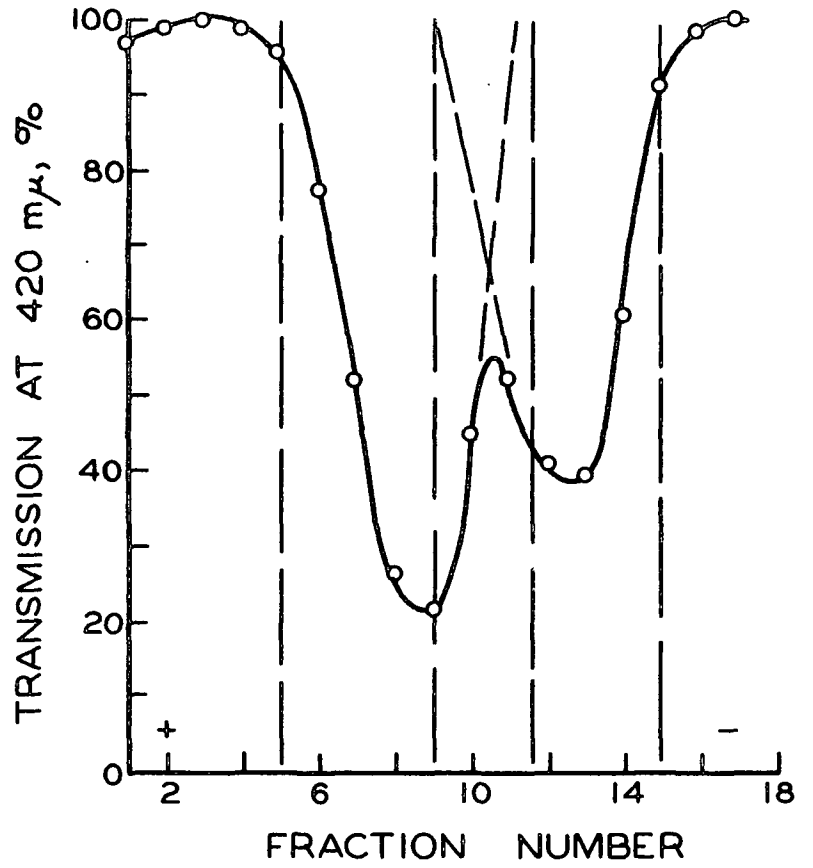

Figure 6. The First Zone Electrophoresis of Crude Pseudolaccase H_3-2

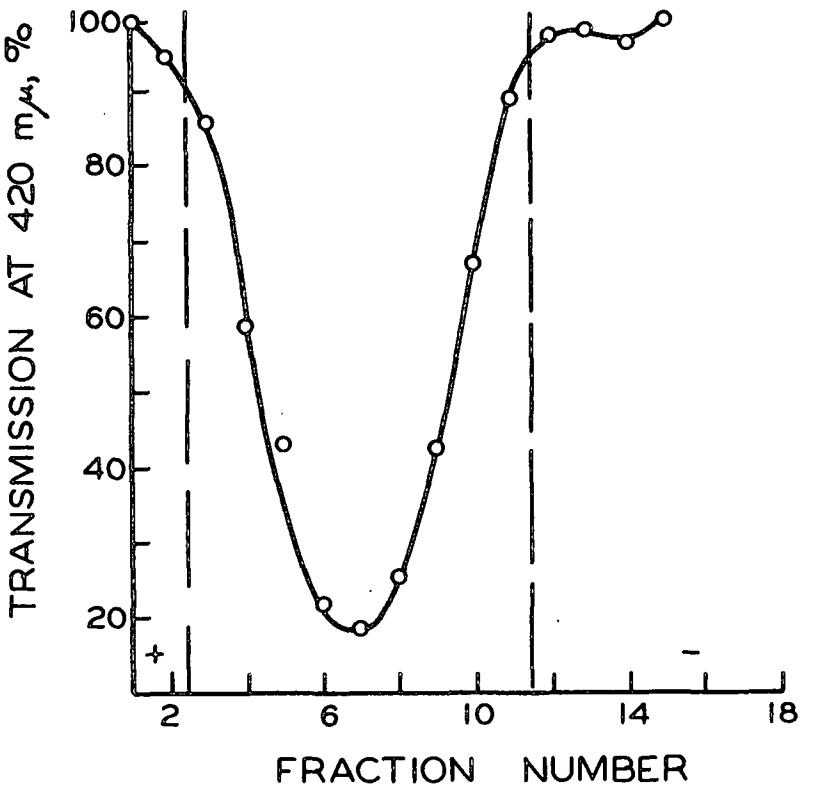

Figure 7. The Third Successive Zone Electrophoresis of Pseudolaccase H_3-2 Yielded Pseudolaccase K

TABLE III

PURIFICATION BY ZONE ELECTROPHORESIS

Preparation	Fraction Rich in Pseudo-laccase Activity	Fraction Rich in Tyrosinase Activity	Fraction with Mixture of Activities	Recovered Without Electrophoresis[a]
Run 8 42 mg. recovered from preliminary countercurrent electrophoresis was dissolved in 3 ml. of buffer solution No. 3.	7.5 mg.	10. mg.	20. mg.	3. mg.
Run 9 119 mg. of preparation H_3-2 was dissolved in 3 ml. of buffer solution No. 3.	20. mg.	20.3 mg.	22. mg.	32. mg.
Run 10 35.1 mg. of preparation H_3-2 was dissolved in 1 ml. of buffer solution No. 3.	15.5 mg.	7.5 mg.	8.4 mg.	2. mg.
Run 11 24.6 mg. recovered from preliminary countercurrent electrophoresis was dissolved in 2 ml. of buffer solution No. 3	6.5 mg.	6. mg.	8. mg.	4. mg.
Run 12 36.4 mg. recovered from preliminary countercurrent electrophoresis was dissolved in 2 ml. of buffer solution No. 4.	16.5 mg. for Run. 14		16.5 mg.	2.5 mg.
Run 13 87.5 mg. were dissolved in 3 ml. of buffer solution No. 4.	38.7 mg.	18. mg.	23. mg.	7. mg.
Run 14 66. mg. were dissolved in 3 ml. of buffer solution No. 4	30. mg. for Run 16		16. mg.	8. mg.
Run 16 68.7 mg. were dissolved in 3 ml. of buffer solution No. 4	55. mg.			5. mg.
Run 17 85. mg. previously subjected to zone electrophoresis twice were dissolved in 3 ml. of buffer solution No. 4.				

Combine these fractions for Run 13

(Analyzed as fractions, see page 44ff.)

[a] Enzyme preparation recovered from cell-loading tools.

ANALYTICAL ELECTROPHORESIS--HETEROGENEITY AND DIFFUSION COEFFICIENTS

Pseudolaccase K was subjected to analytical electrophoresis for quantitative characterization of heterogeneity and diffusion coefficients and electrophoretic migration rates. Pseudolaccase K was insoluble at the isoelectric pH of 3.55; therefore, experiments were performed at pH 5, 7, and 9 in buffer solutions 5, 4, and 3, respectively. Figure 8 displays typical electrophoretic patterns. Table IV summarizes the experimental conditions and the results which emanate from Fig. 9, a plot of apparent diffusion coefficient vs. time according to Alberty (56) and others (60,61).

One principal component, accounting for 90% or more of preparation K, can be seen in Fig. 8 at the 3 pHs investigated. Enantiography of the ascending and descending schlieren patterns was good at pHs 7 and 9 indicating that pH and concentration gradients were very small. At pH 5, the enantiography was excellent. The enantiography indicated that all phenomena, except diffusion and electrophoretic mobility, were minimized and a significant heterogeneity coefficient characteristic of pseudolaccase K could be determined. The 3 component nature of pseudolaccase K was not detected at this time because the combination of component concentration and electrophoretic mobility differences was effectively diminished by the low field strength and relatively short times employed in electrophoresis spreading experiments.

ULTRACENTRIFUGAL ANALYSIS

Pseudolaccase K, in phosphate buffer solution (No. 4), was subjected to measurements of the sedimentation velocity coefficient using the

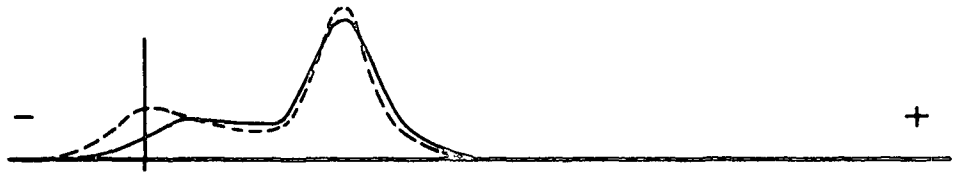

Figure 8a. Magnified Tracing of the Electrophoretic Pattern (Solid Line, Descending Boundary; Dotted Line, Ascending Boundary) of Pseudo-laccase K at 0.5% in Buffer Solution No. 4 [The Field Strength was 2.55 Volts/Cm., the Heat Dissipation was 0.032 Watts/ml., and the Time was 16 Min. The (+) and (-) Indicate the Anode and Cathode, Respectively.]

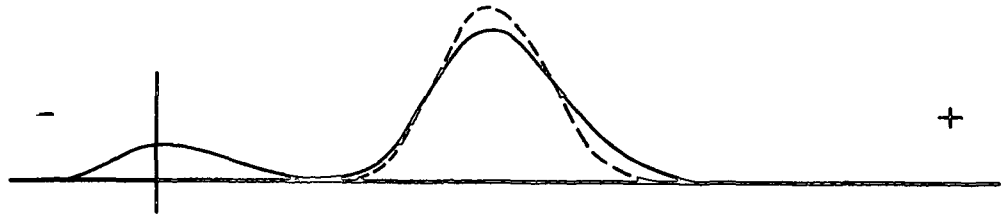

Figure 8b. As for Fig. 8a Except That the Time was 45 Min.

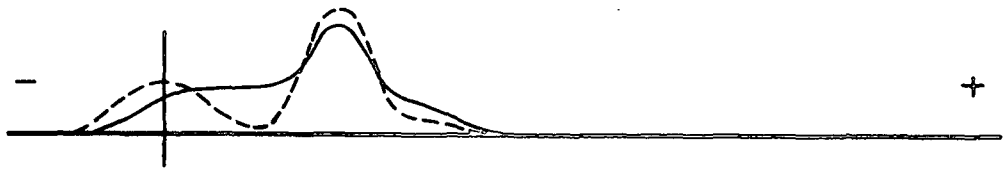

Figure 8c. Magnified Tracing of the Electrophoretic Pattern (Solid Line, Descending Boundary; Dotted Line, Ascending Boundary) of Pseudo-laccase K at 0.45% in Buffer Solution No. 3 [The Field Strength was 2.30 Volts/Cm., the Heat Dissipation was 0.0286 Watts/ml., and the Time was 20 Min. The (+) and (-) Indicate the Anode and Cathode, Respectively.]

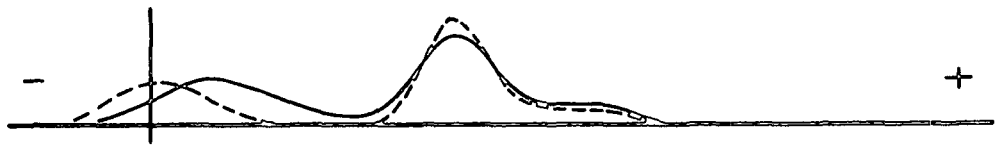

Figure 8d. As for Fig. 8c Except That the Time was 46 Min.

Figure 8e. As for Fig. 8c Except That the Time was 113 Min.

Figure 8f. As for Fig. 8c Except That the Time was 205 Min. (This was 90 Minutes After Reversing the Current)

Figure 8g. Magnified Tracing of the Electrophoretic Pattern (Solid Line, Descending Boundary; Dotted Line, Ascending Boundary) of Pseudolaccase K at 0.4% in Buffer Solution No. 5 [The Field Strength was 1.42 Volts/cm., the Heat Dissipation Was 0.0089 Watts/Ml., and the Time Was 52 min. The (+) and (-) Indicate the Anode and Cathode, Respectively.]

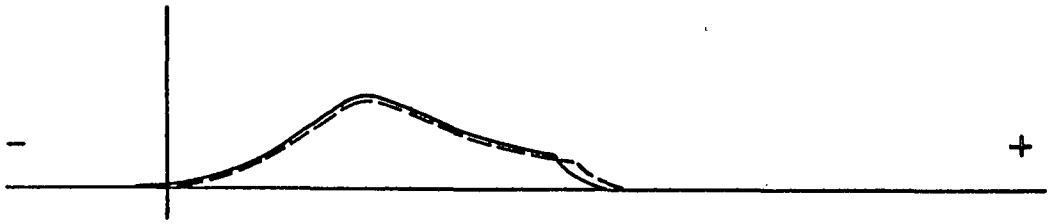

Figure 8h. As for Fig. 8g Except that the Time Was 112 Min.

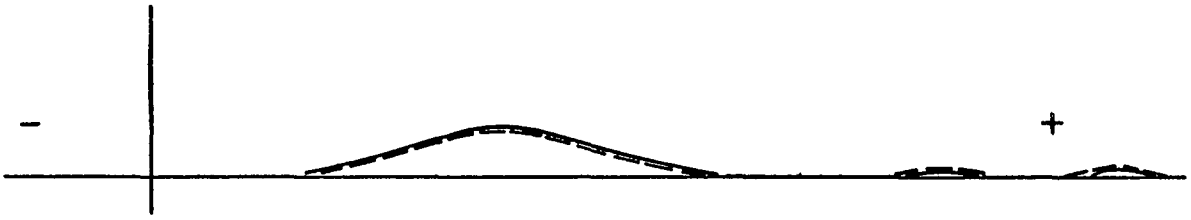

Figure 8i. As for Fig. 8g Except that the Time Was 232 Min.

Figure 8j. As for Fig. 8g Except that the Time Was 412 Min. (This Was 172 Minutes After Reversing the Current)

TABLE IV

HETEROGENEITY AND DIFFUSION COEFFICIENTS FOR PSEUDOLACCASE K

(Experimental conditions and results)

pH	Temperature, °C.	Enzyme Concentration, %	Field Strength, volts/cm.	Heat Dissipation, watts/ml.	Heterogeneity Coefficient, sq.cm./volt/sec.	Diffusion Coefficient, sq.cm./sec.
5	0.5	0.4	1.42	0.0089	1.02×10^{-5}	7.9×10^{-7}
7	0.5	0.5	2.55	0.032	1.73×10^{-5}	7.9×10^{-7}
9	0.5	0.45	2.30	0.0286	1.08×10^{-5}	12.5×10^{-7}[a]

[a]This value was inconsistent with 3 identical values determined independently; the reason remained unexplained.

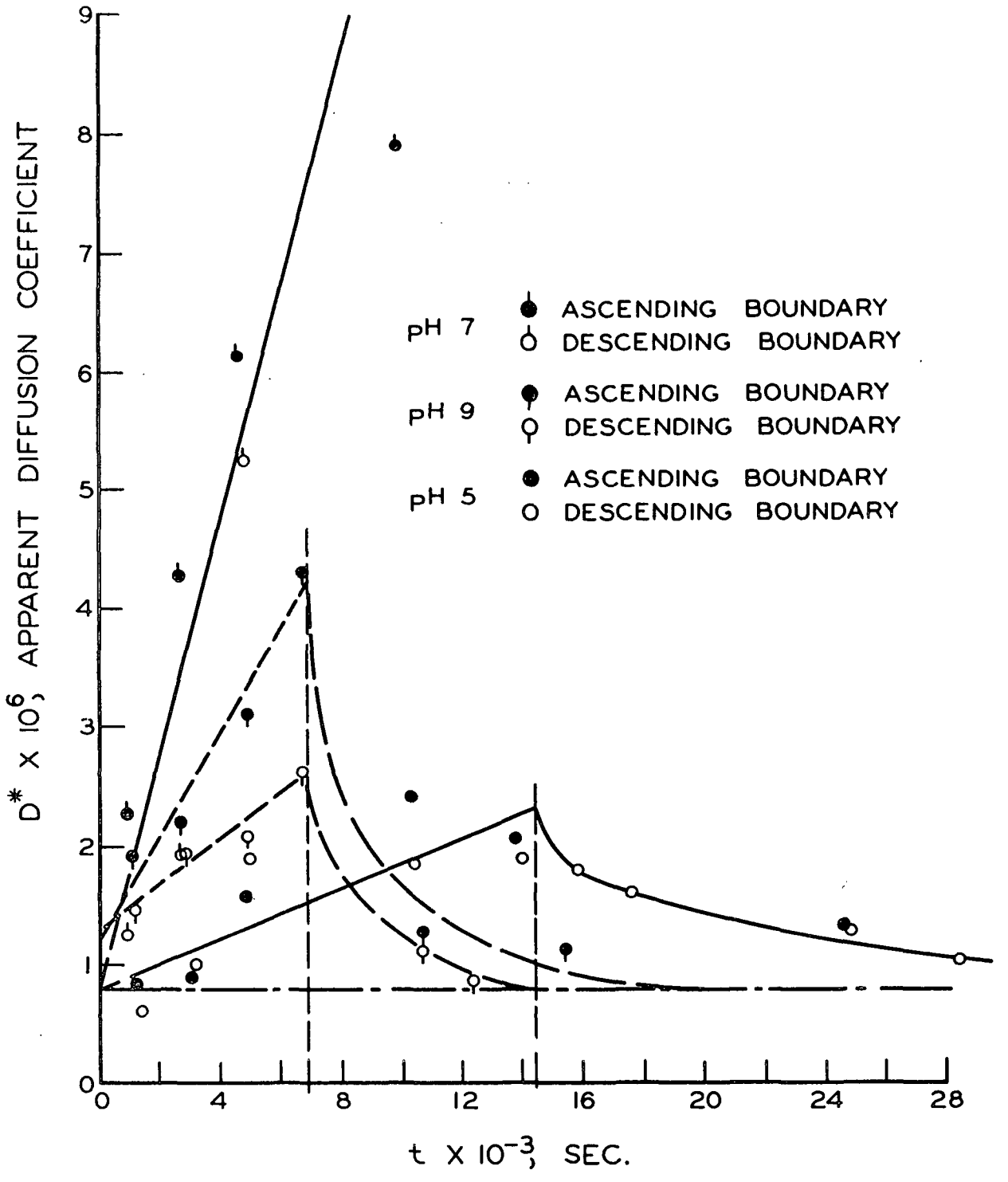

Figure 9. Apparent Diffusion Coefficient vs. Time for Electrophoresis Spreading of Pseudolaccase K. (See the Text and Table IV for Complete Details; The Straight Lines were Positioned by the Method of Least-Squares)

Beckman/Spinco Model E ultracentrifuge[a] with automatic temperature control. The sedimentation, as measured by the radial movement of the maximum ordinate of the concentration gradient curve, is plotted vs. time in Fig. 10. The slope yielded an observed sedimentation coefficient, s, equal to 3.82 Svedbergs by using Equation (2), page 12. The weight average molecular weight equaled 35,000 when a partial specific volume equal to 0.663 ml./g. and a diffusion coefficient of 7.9×10^{-7} sq.cm./sec. were substituted into Equation (1), page 12.

A single, nearly Gaussian, concentration gradient curve (52) existed in a synthetic boundary cell after 60 minutes of ultracentrifugation of pseudolaccase K. An earlier sedimentation velocity run on pseudolaccase H_3-2 yielded 2 peaks. Their calculated molecular weights were about 100,000 and 40,000 which agreed well with published molecular weights for tyrosinase (33) and the above molecular weight for pseudolaccase K, respectively.

An estimate of the diffusion coefficient of pseudolaccase K was made from data obtained in the above-described sedimentation velocity experiment. A plot of the second moment of the concentration gradient curve vs. time appears in Fig. 11, where the slope of the straight line equals 2D (see Appendix VI, page 105). The diffusion coefficient equaled 7.9×10^{-7} sq. cm. /sec. which is the same as those determined by analytical electrophoresis at pH 5 and 7.

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[a] Beckman/Spinco Ultracentrifuge Technical Manual--Model E. Palo Alto, California, Beckman Instruments, Inc., 1959.

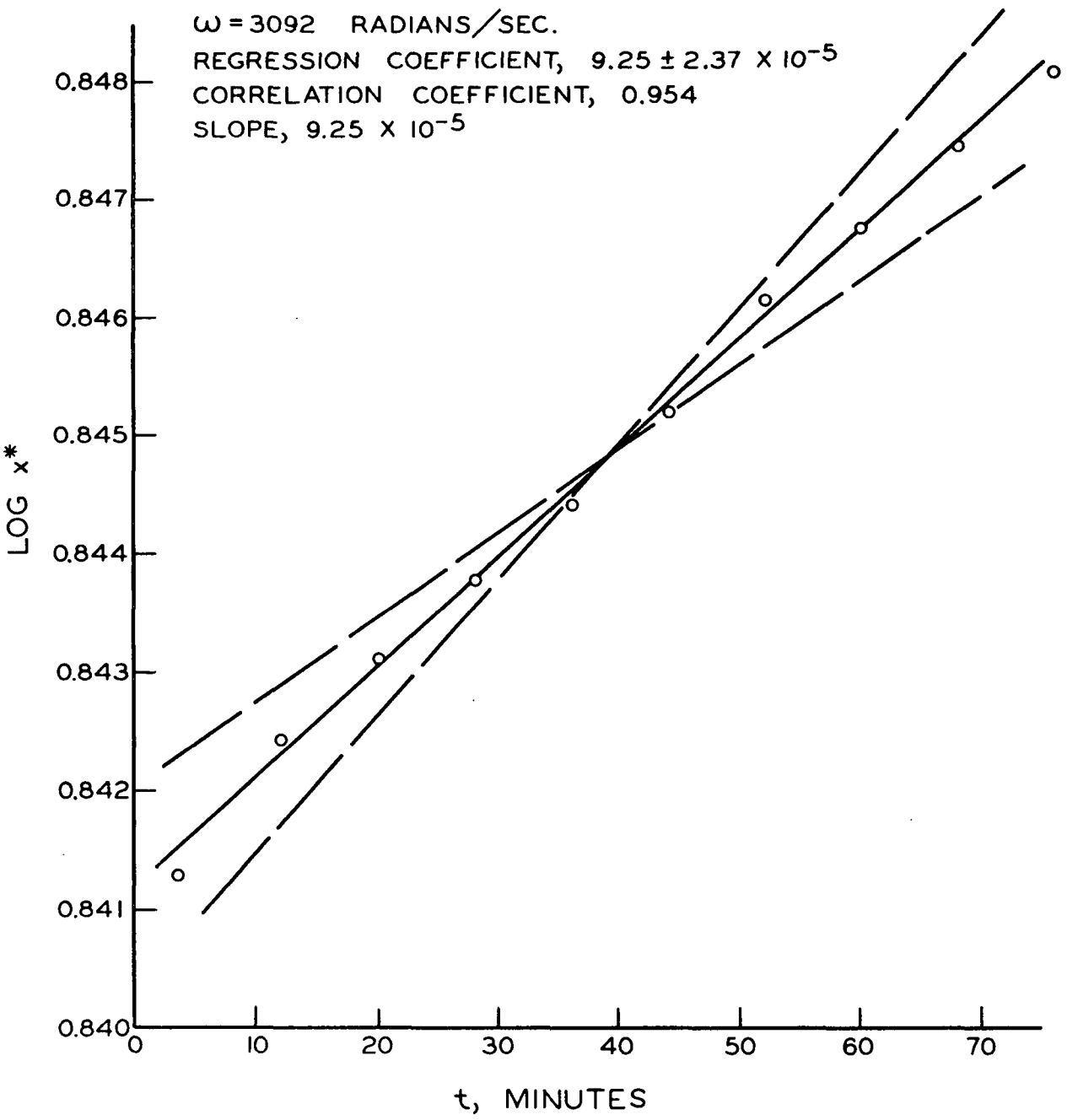

Figure 10. Change in the Radius of the Maximum Ordinate with Time (Pseudolaccase K in Phosphate Buffer Solution, No. 4, at 20°C.)
*$\underline{x}$ was the distance between the maximum ordinate and its axis of rotation in cm.

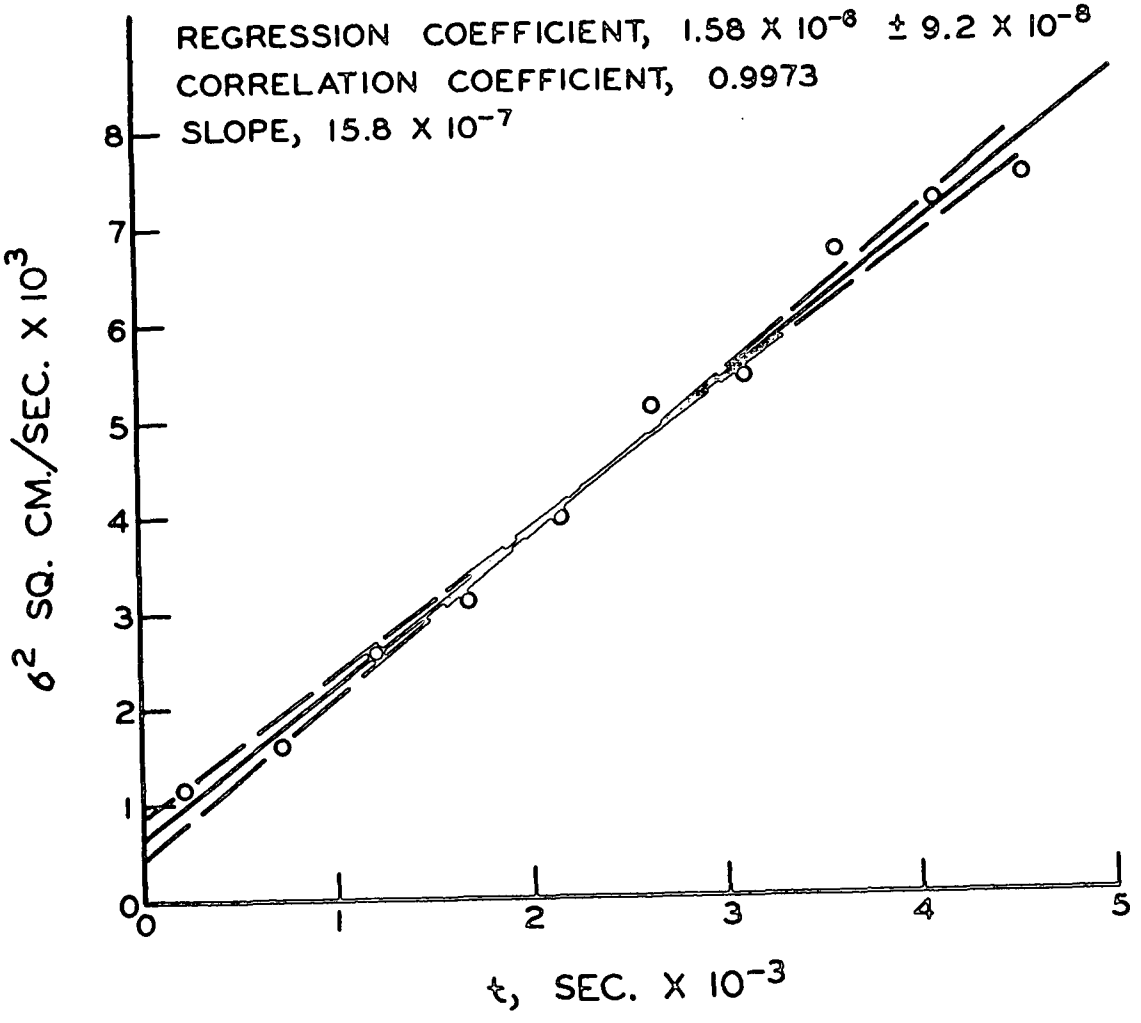

Figure 11. Diffusion Coefficient by Sedimentation Velocity Experiment. (One-Half the Slope Equals the Diffusion Coefficient for Pseudolaccase K)

Pseudolaccase K was subjected to a sedimentation equilibrium experiment at relatively low speed (ca. 10,000 r.p.m.) for 4 days. Equilibrium was essentially complete after 2.5 days. Figure 12 is a plot of log $(1/x)(dc/dx)$ vs. x^2 which yields a straight line for preparation homogeneous with respect to molecular weight. Pseudolaccase K possessed a distribution of molecular weights.

PARTIAL SPECIFIC VOLUME

Figure 13 graphically presents the data which were used in Equation (4) to calculate the partial specific volume of pseudolaccase K.

$$\bar{V}_{app.} = \frac{1}{\rho_o} - \frac{1}{c}\left(\frac{\rho - \rho_o}{\rho_o}\right) \qquad (4)$$

The densities of solution and solvent are respectively, ρ and ρ_o; c is the concentration of solute in grams per milliliter of solution. The raw data are in Table V. The average of three determinations gave 0.663 ml./g. as the partial specific volume of pseudolaccase K.

ABSORPTION SPECTRUM OF PSEUDOLACCASE K

The absorption spectrum of pseudolaccase K was measured using a Beckman DK-2 ratio recording spectrophotometer. Figure 14 shows that there were no absorption maxima, but a shoulder existed at 280 mμ. The spectrum of Chinese lacquer tree (R. vernicifera) laccase as determined by Nakamura (43) is also presented. Both spectra were determined in approximately the same concentration of phosphate buffer at pH 7.0.

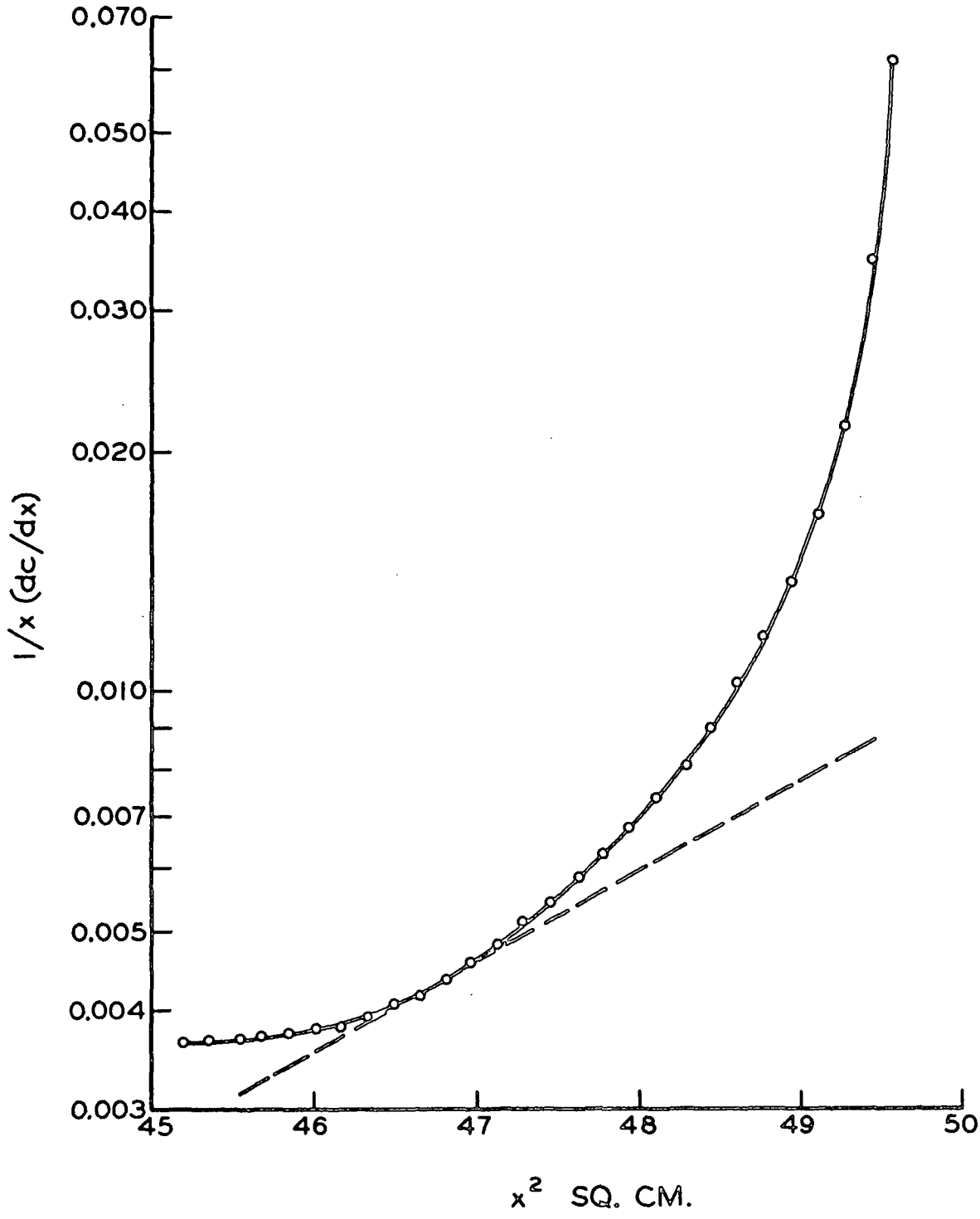

Figure 12. Sedimentation Equilibrium for Pseudolaccase K

(the Dotted Line has the Correct Slope for a Molecular Weight of 35,000)

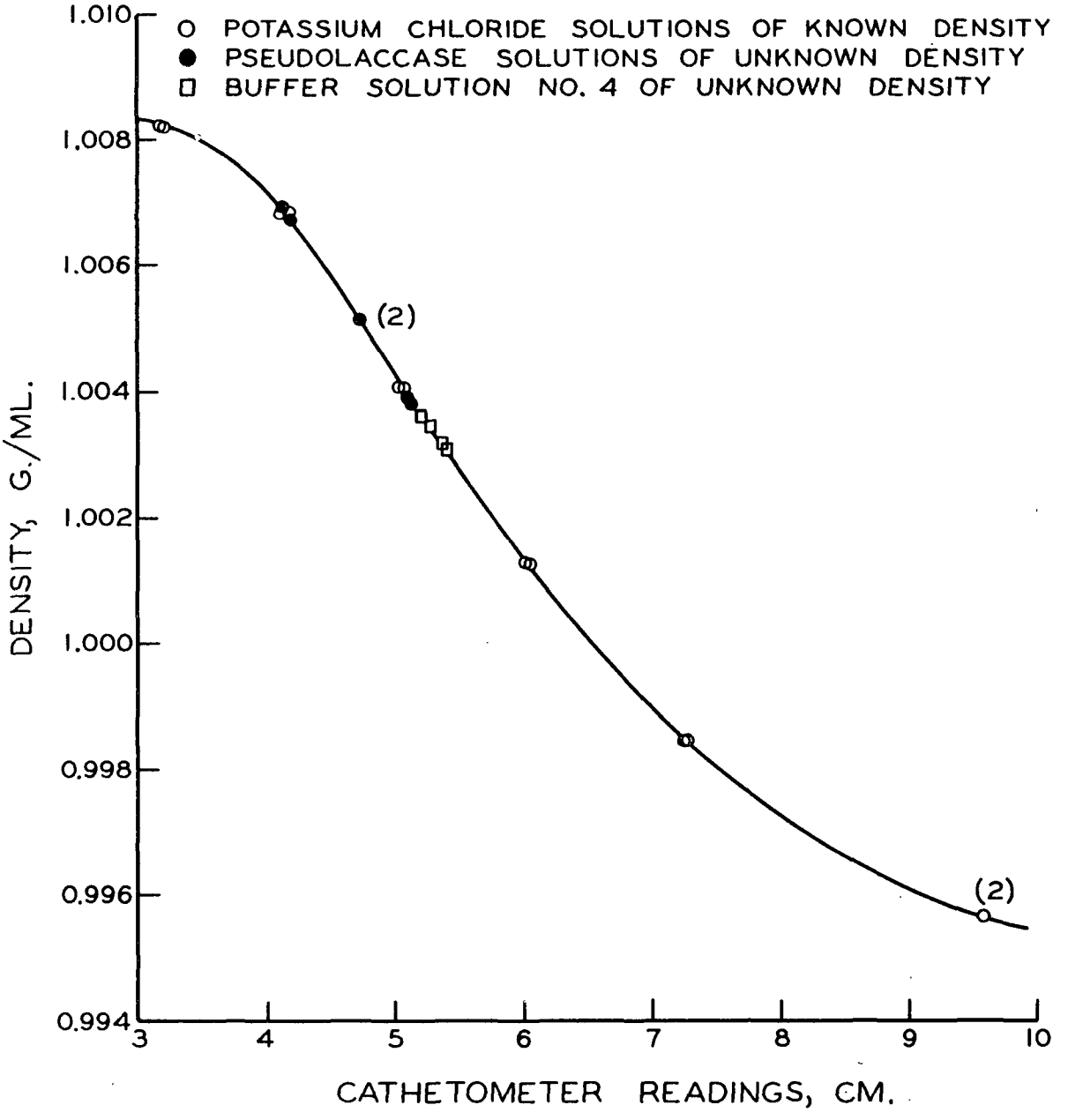

Figure 13. Density Gradient Column Calibration and Use at 30°C.

TABLE V

PARTIAL SPECIFIC VOLUME OF PSEUDOLACCASE K

(Density gradient column data at 30°C.)

Solution	Known Density	Solution Concentration, g./ml.	Cathetometer Reading[a], cm.	Density Found	Partial Specific Volume, $\bar{V}$
KCl					
1	1.00823		3.400		
			3.360		
2	1.00685		4.110		
			4.193		
3	1.00407		5.043		
			5.063		
4	1.00128		6.022		
			6.040		
5	0.99847		7.290		
			7.273		
6	0.99565		9.556		
(Water)			9.572		
Enzyme					
1		0.010439	4.109	1.00679	0.660
			4.177		
2		0.005365	4.730	1.00509	0.655
			4.736		
3		0.001788	5.115	1.00383	0.674
			5.149		
Buffer			5.410		
No. 4			5.368	1.00325	
			5.271		
			5.204		

[a]All readings were made 90 min. after drop was placed in the column. Duplicate readings represent duplicate drops. (A study of drop position with time indicated all drops were stable at 90 min.)

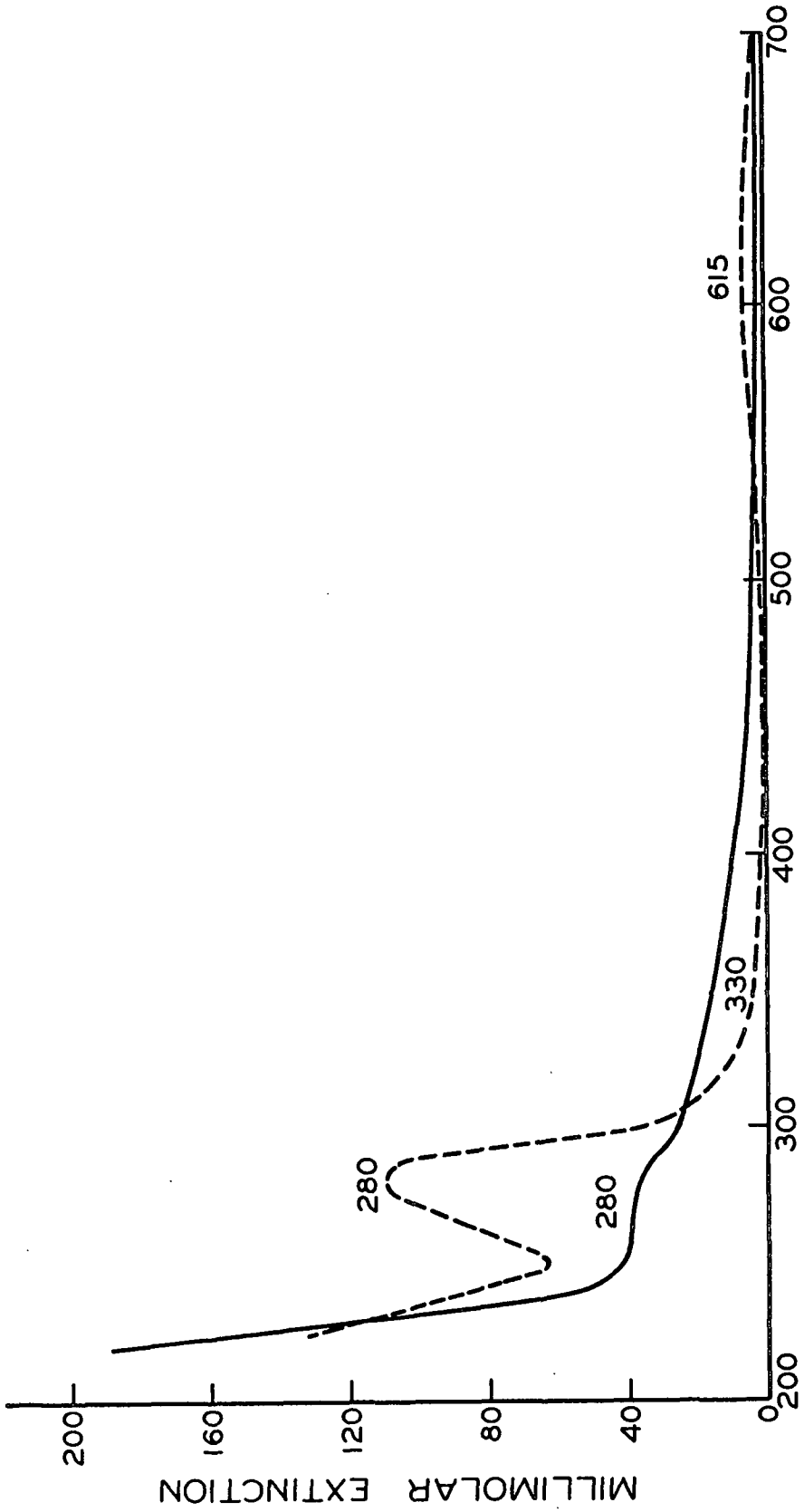

Figure 14. Absorption Spectrum of Pseudolaccase K in Phosphate Buffer, pH 7 and 0.1M, ——; Absorption Spectrum of Laccase in Phosphate Buffer, pH 7.0 and Ionic Strength, 0.2, – – – – (43)

CHEMICAL PROPERTIES OF PSEUDOLACCASE K

After wet ashing, the copper content of several fractions of pseudolaccase K (see the next section) was measured colorimetrically at 479 mμ by the bathocuproine method (71) using n-hexanol as solvent. No trend was noted for the copper content of the fractions. The average was 0.19% copper on a dry-weight basis. The nitrogen content, which was measured by the Dumas method, was 3.94% for a whole preparation. Determinations on fractions of pseudolaccase K gave a weight average of 5.24% nitrogen. The Molisch reaction, tried on an enzyme solution, was positive, and suggested the presence of combined carbohydrate. A hydrolyzate of pseudolaccase K was spotted on Whatman No. 1 chromatography paper, developed with either EAPW or EAPWA, and sprayed with p-A. Galactose and xylose were detected in a ratio of about 10:1. Assuming the protein component of the enzyme was 16% nitrogen, the carbohydrate component was estimated at 67% of the preparation weight. The chromatographic results did not seem to account for as much as 67% carbohydrate in the enzyme based on the color intensity of the spots on a p-A sprayed chromatogram. At least 9 amino acids comprised the protein part of the enzyme.

CHARACTER OF AN ELECTROPHORETICALLY FRACTIONATED PSEUDOLACCASE K

Pseudolaccase H_3-2, the crude product of the methanol fractionation of an aqueous extract of mushroom stem bases, was subjected to three successive zone electrophoresis experiments. After the third zone electrophoresis run of 30 hours at 30 ma. (field strength = 2.19 volts/cm.), the catechol-active fractions were not combined to yield preparation K, as was

done previously, but each fraction was characterized individually. Figure 15 and Table VII summarize the results.

Weight and specific activity distributions correlated well to indicate that three components comprised pseudolaccase K. A comparison of the electrophoretic mobilities of the three components with the electrophoretic mobilities of components II, III, and IV of crude preparation H_3-2 (Fig. 4, page 24) indicated that these components were, indeed, the same. See Table VI. The nitrogen and copper analyses and the spectrophotometrically measured catechol activity did not reveal the heterogeneous nature of pseudolaccase K. Vanillin was not oxidized at all by the fractions of pseudolaccase K, while ascorbic acid was oxidized at a nearly constant rate by the three fractions characterized. Component II catalyzed the oxidation of hydroquinone, coniferyl alcohol, ferulic acid, ascorbic acid, and catechol. Components III and IV catalyzed the oxidation of hydroquinone, catechol, coniferyl alcohol, ferulic acid, syringaldehyde, and ascorbic acid. Component III was considerably more active than II or IV.

TABLE VI

ELECTROPHORETIC MOBILITIES OF COMPONENTS
II, III, AND IV OF PSEUDOLACCASE K

(Buffer solution No. 4 was used at 0.5°C.)

Component	Electrophoretic Mobility, sq. cm./volt-second	
	K (Fig. 15)	H_3-2 (Fig. 4)
II	0.51×10^{-4}	0.48×10^{-4}
III	0.66×10^{-4}	0.63×10^{-4}
IV	0.96×10^{-4}	0.85×10^{-4}

TABLE VII

PSEUDOLACCASE K FRACTION CHARACTERIZATION

(Preparation K is equivalent to the sum of fractions 2 through 11)

Fraction	Weight, mg.	Volume, ml.	Weight Distribution, mg./ml.	Nitrogen, %	Copper, %	Specific Activity, $(Q_{O_2}) \times 10^{-4}$ HQ[a]	CA[b]	FA[c]	SYR[d]	VAN[e]	AA[f]
1	6.882	11.5	0.598	1.34	0.162	--	--	--	--	--	--
2	7.775	7	1.111	1.90	0.226	1.53	0.27	0.00	0.00	--	1.18
3	7.777	6	1.296	2.87	0.182	2.94	0.74	0.26	0.00	0.00	--
4	6.458	5	1.292	4.43	0.172	3.36	0.68	0.53	0.00	0.00	--
5	5.375	4	1.344	5.34	0.194	6.08	2.22	1.36	1.05	0.00	--
6	4.538	3	1.513	6.19	--	7.71	3.12	1.83	1.47	--	1.01
7	4.810	3	1.603	6.64	0.248	7.31	3.27	2.21	1.87	0.00	--
8	6.657	4	1.664	7.34	0.186	3.94	2.97	2.28	1.90	0.00	--
9	6.979	5	1.396	7.54	0.186	2.32	1.93	1.10	0.88	0.00	--
10	7.622	6	1.270	6.79	0.100	1.53	0.99	0.72	0.43	0.00	--
11	7.641	7	1.092	4.74	0.220	0.76	0.22	0.00	0.00	--	0.94
12	4.879	7	0.697	1.93	0.000	--	--	--	--	--	--
13	3.475	7	0.496	0.89	--	--	--	--	--	--	--
14	3.646	7	0.521	0.97	--	--	--	--	--	--	--
Weight average, Fractions 2 through 11				5.24	0.188	3.34					
Preparation K				3.94		3.53					

[a] Hydroquinone, 10 mg. and sodium sulfite, 6 mg. per flask; buffer solution No. 6, 25°C.

[b] Coniferyl alcohol, 20 μM per flask; buffer solution No. 6, 25°C.

[c] Ferulic acid, 20 μM per flask; buffer solution No. 6, 25°C.

[d] Syringaldehyde, 20 μM per flask; buffer solution No. 6, 25°C.

[e] Vanillin, 20 μM per flask; buffer solution No. 6, 25°C.

[f] Ascorbic acid, 20 μM per flask; buffer solution No. 6, 25°C.

REACTION RATE STUDIES

The Warburg apparatus was used successfully to measure oxygen absorption by the p-coumaryl, sinapyl, and coniferyl alcohol-pseudolaccase K systems in 0.05M phosphate buffer solutions at pH 6.2, 6.7, 7.2, and 7.7. The method of Van Slyke, et al. (67) was successfully applied to the β-C^{14}-coniferyl alcohol-pseudolaccase K system under the same experimental conditions. The rates of formation of dehydrodiconiferyl alcohol, pinoresinol, and guaiacylglycerol-β-coniferyl ether were also measured in the system containing radiocarbon. Initial reaction rates were obtained as a function of substrate concentration and pH by plotting μl. of oxygen absorbed, substrate alcohol reacted, or product formed vs. time as in Fig. 30 to 57 in Appendix IX. Tables XIII to XL contain the numerical data.

The initial reaction rate data were used to construct Fig. 16 to 22 by the method of Lineweaver and Burk (64). The reciprocal of the intercept on a Lineweaver-Burk plot equaled the maximum velocity, V, of the reaction for a given set of conditions. As shown in Appendix VII, $V = k_3(E_t)$, and since the total enzyme concentration, (E_t), was constant, the magnitude of V was a good index of the over-all reaction velocity; i.e., the rate of disappearance of substrate or appearance of products. The effect of pH on V is shown in Fig. 23 to 25. Table VIII contains the numerical data.

For the experimental conditions employed in this research, the straight lines on the Lineweaver-Burk plots indicated that all reaction rates were first order and the enzyme-substrate complex theory of Michaelis-Menten

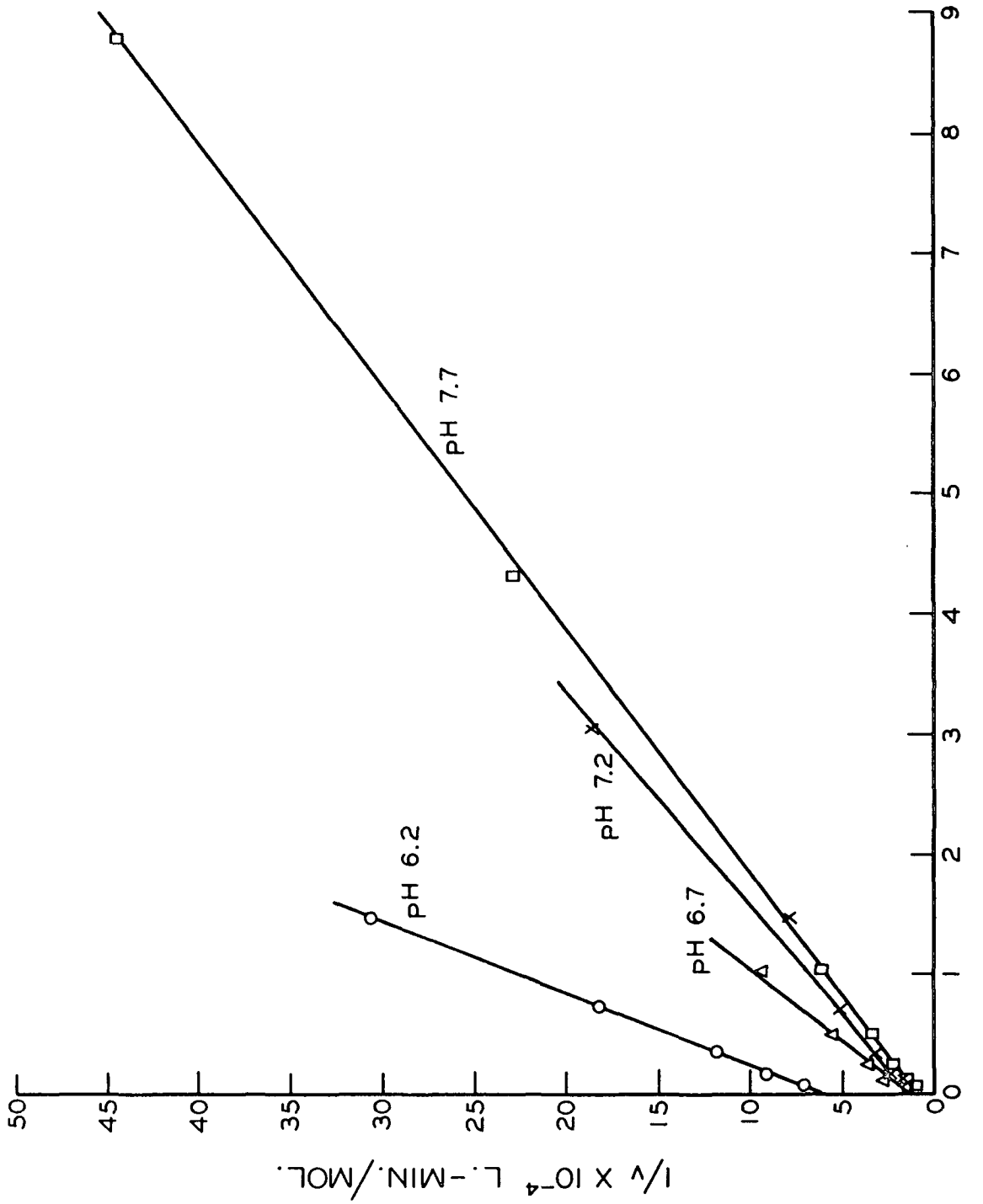

Figure 16. Effect of pH on the Rate of Reaction in the p-Coumaryl Alcohol-Pseudolaccase K System as Plotted by the Method of Lineweaver and Burk (64) (All Buffers were 0.05M Phosphate; Temperature was 25°C. Data by Oxygen Absorption)

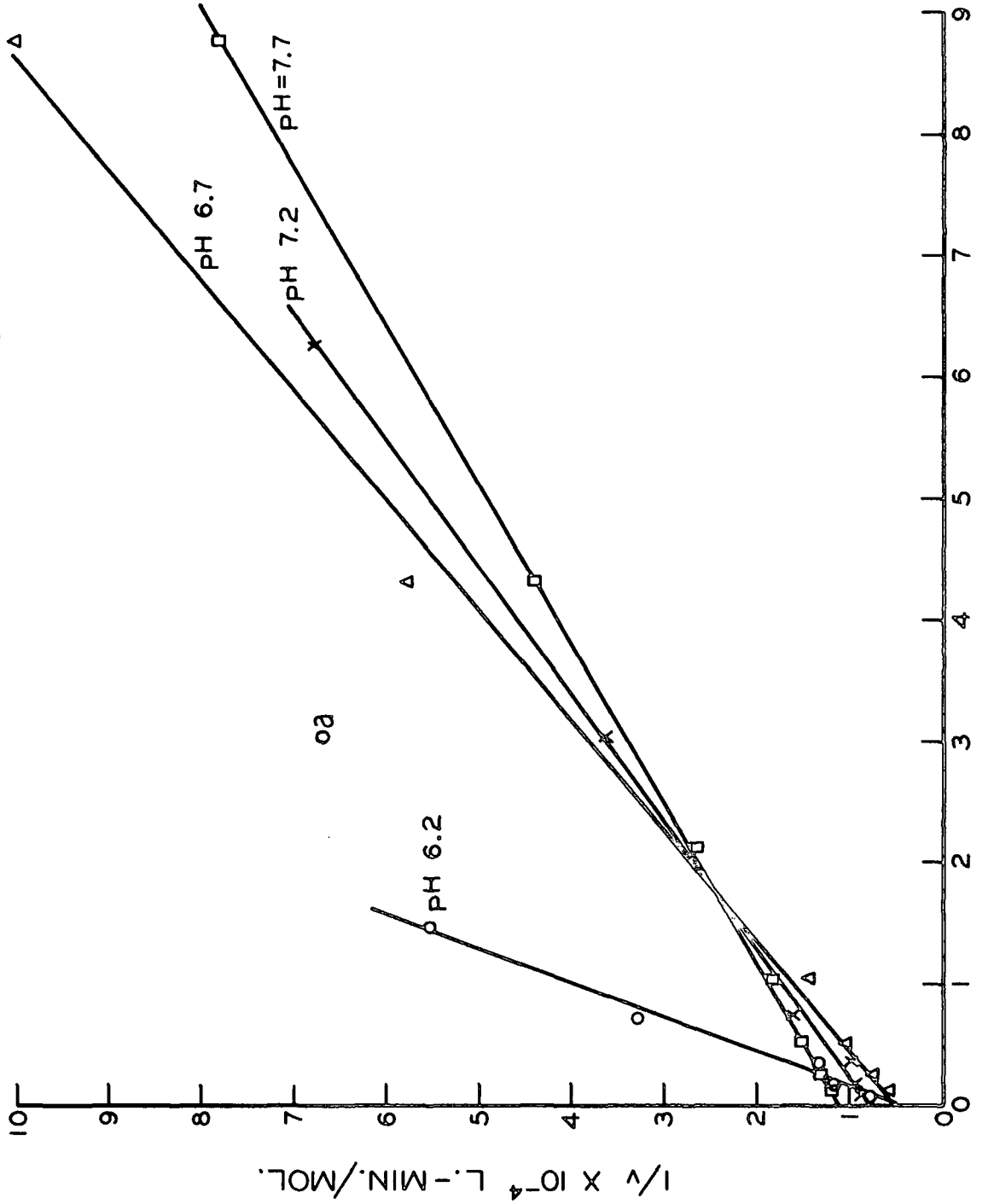

Figure 17. Effect of pH on the Rate of Reaction in the Sinapyl Alcohol-Pseudolaccase K System as Plotted by the Method of Lineweaver and Burk (64) (All Buffers were 0.05M Phosphate; Temperature was 25°C. Data by Oxygen Absorption) [a]Two points for the sinapyl alcohol-pseudolaccase K system at pH 6.2 were inconsistant with the straight-line correlations generally found. The inconsistancy was not explained.

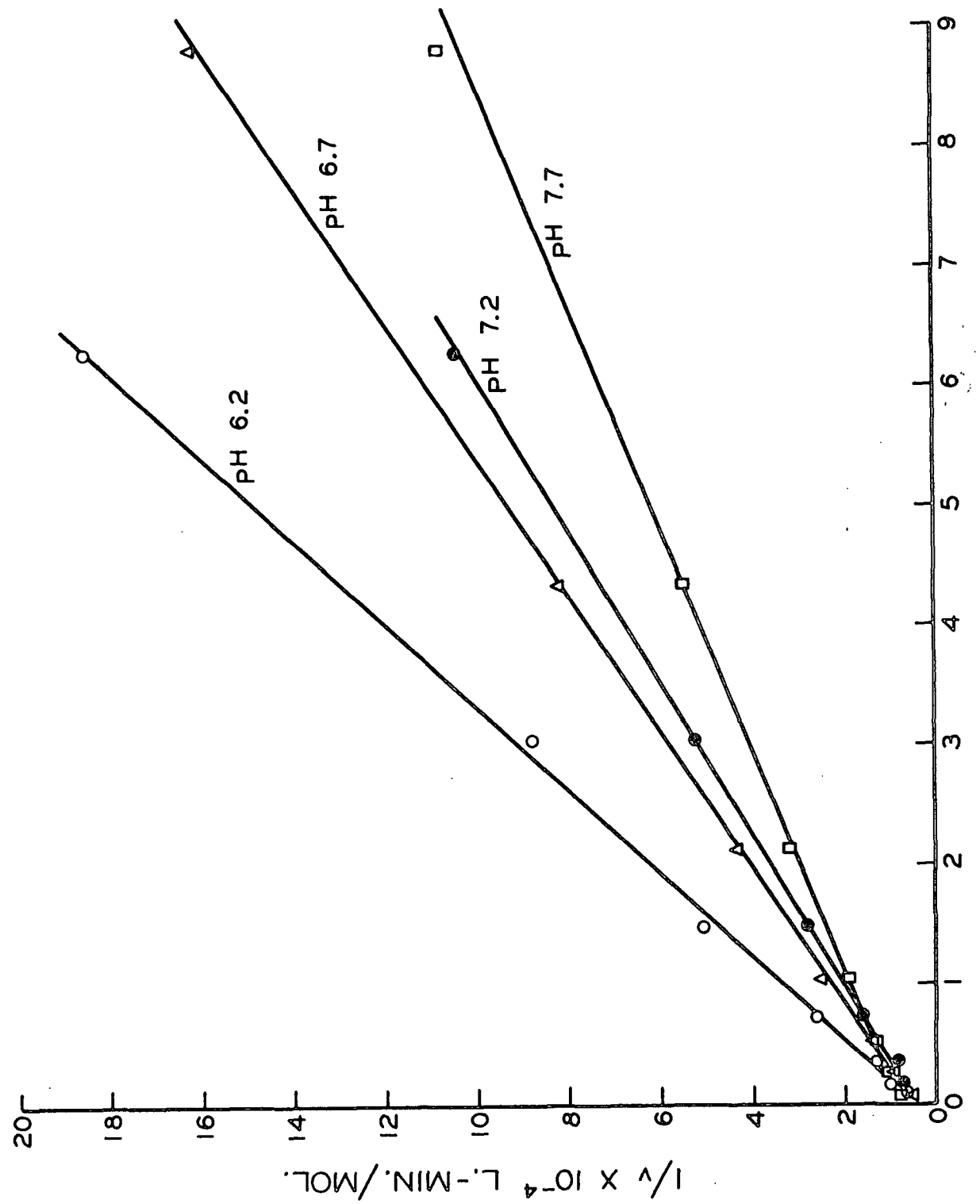

Figure 18. Effect of pH on the Rate of Reaction in the Coniferyl Alcohol-Pseudolaccase K System as Plotted by the Method of Lineweaver and Burk (64)
(All Buffers were 0.05M Phosphate; Temperature was 25°C.
Data by Oxygen Absorption)

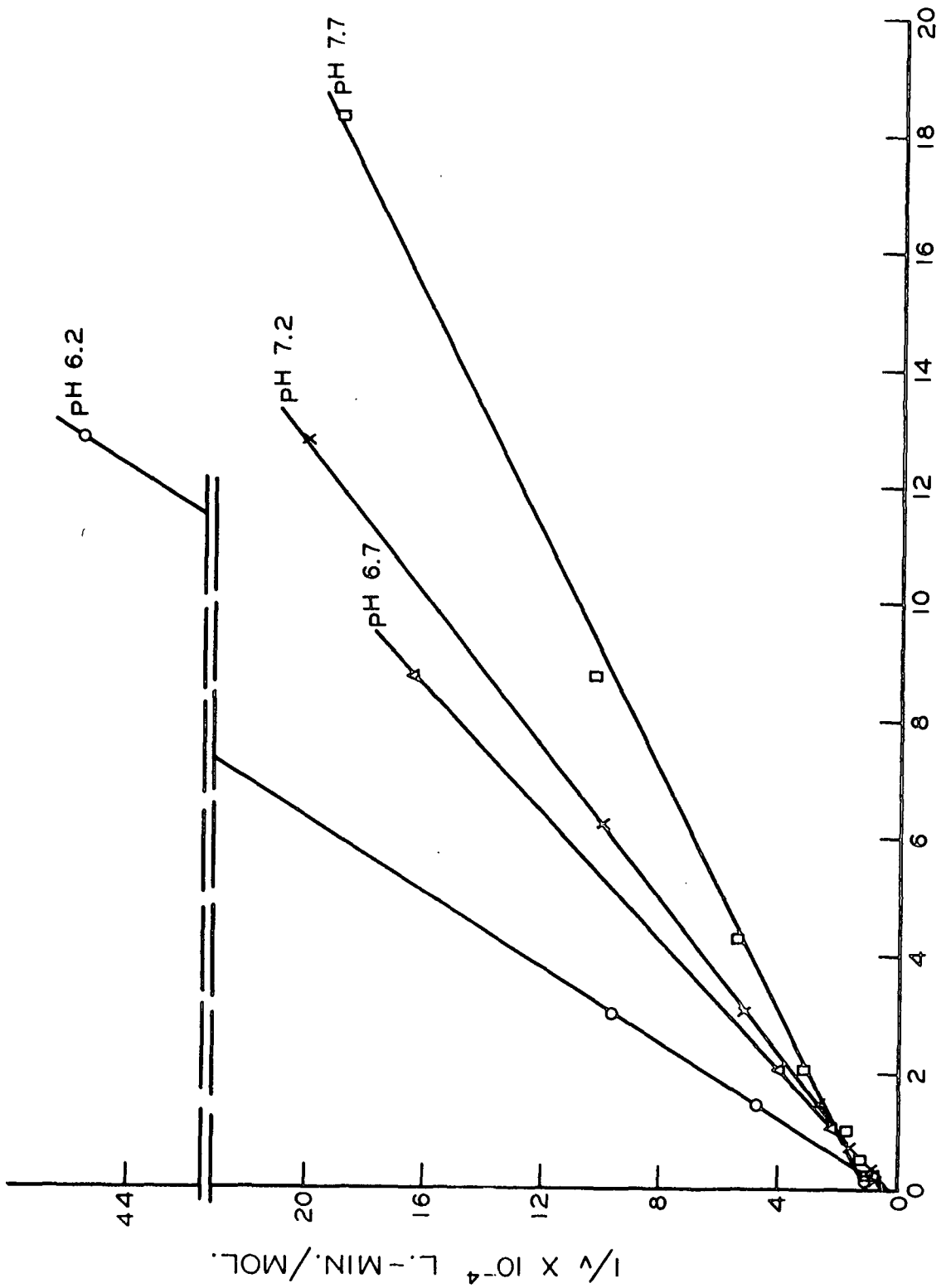

Figure 19. Effect of pH on the Rate of Reaction in the Coniferyl Alcohol-Pseudolaccase K System as Plotted by the Method of Lineweaver and Burk (64) (All Buffers were 0.05M Phosphate; the Temperature Was 25°C.; Data Collected by C^{14} Counting)

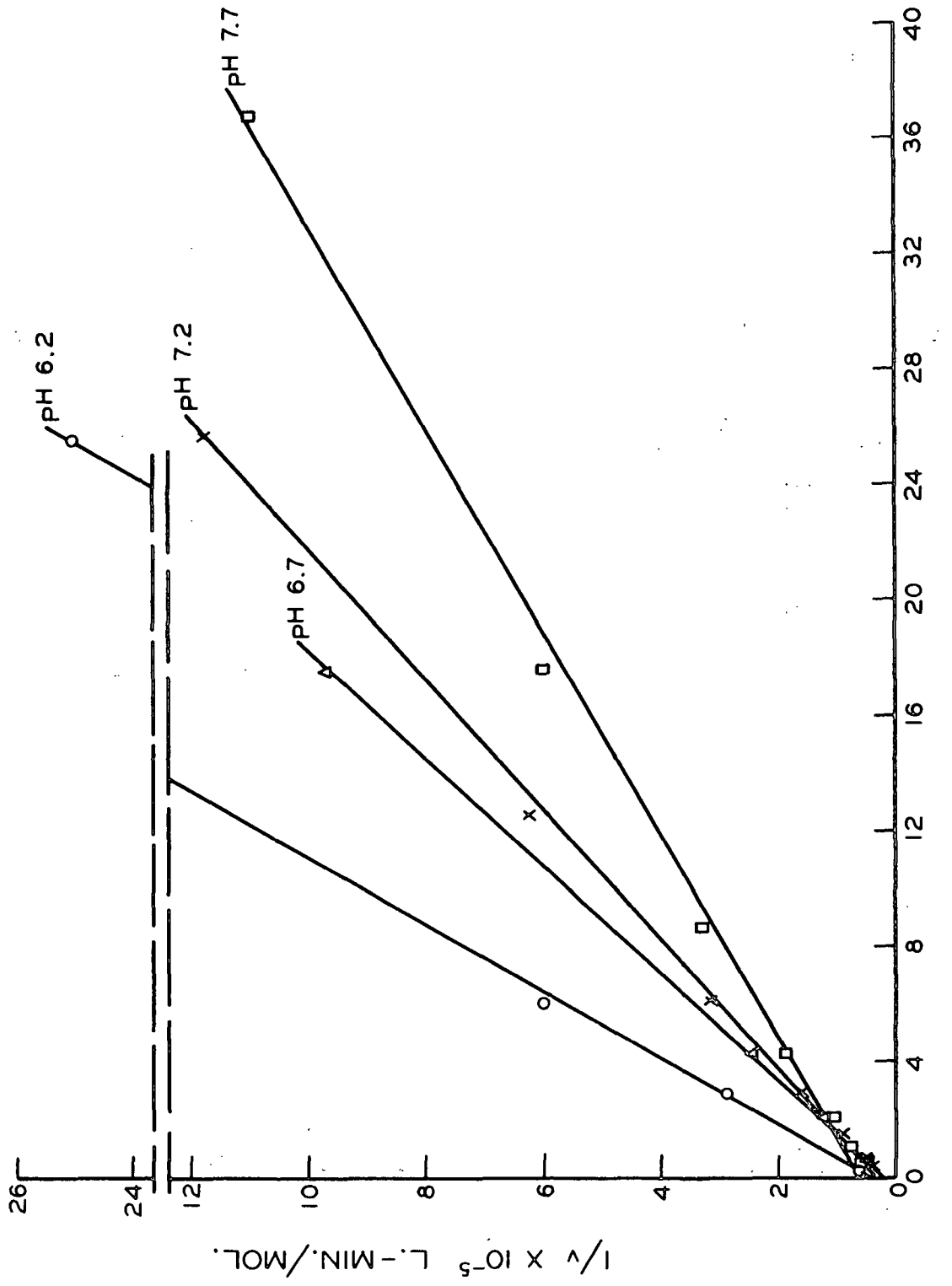

Figure 20. Effect of pH on the Formation of Dehydrodiconiferyl Alcohol in the Coniferyl Alcohol-Pseudolaccase K System as Plotted by the Method of Lineweaver and Burk (64)
(All Buffers Were 0.05M Phosphate; the Temperature Was 25°C.; Data Collected by C^{14} Counting)

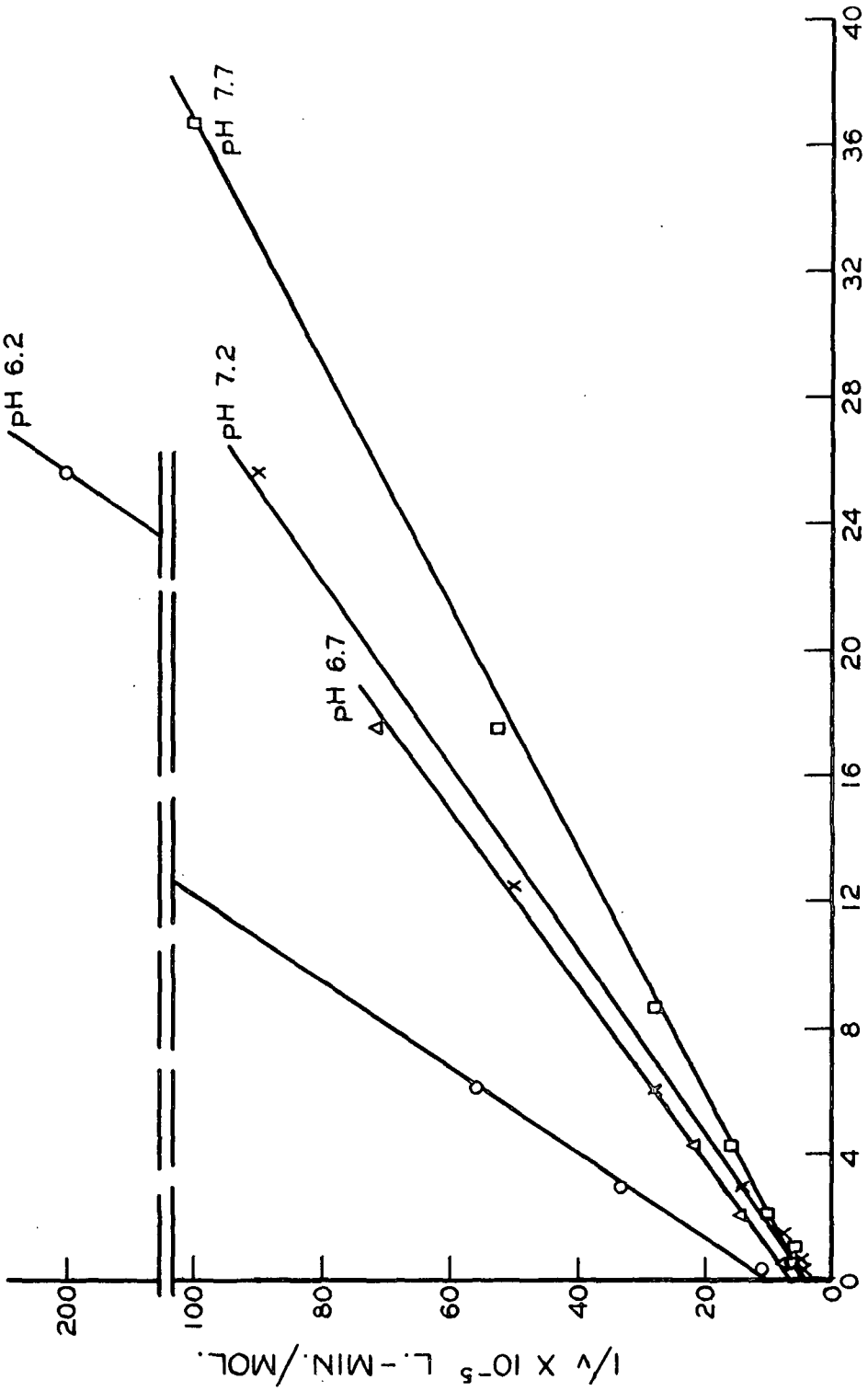

Figure 21. Effect of pH on the Formation of dl-Pinoresinol in the Coniferyl Alcohol-Pseudolaccase K System as Plotted by the Method of Lineweaver and Burk (64) (All Buffers Were 0.05M Phosphate; the Temperature Was 25°C.; Data Collected by C^{14} Counting)

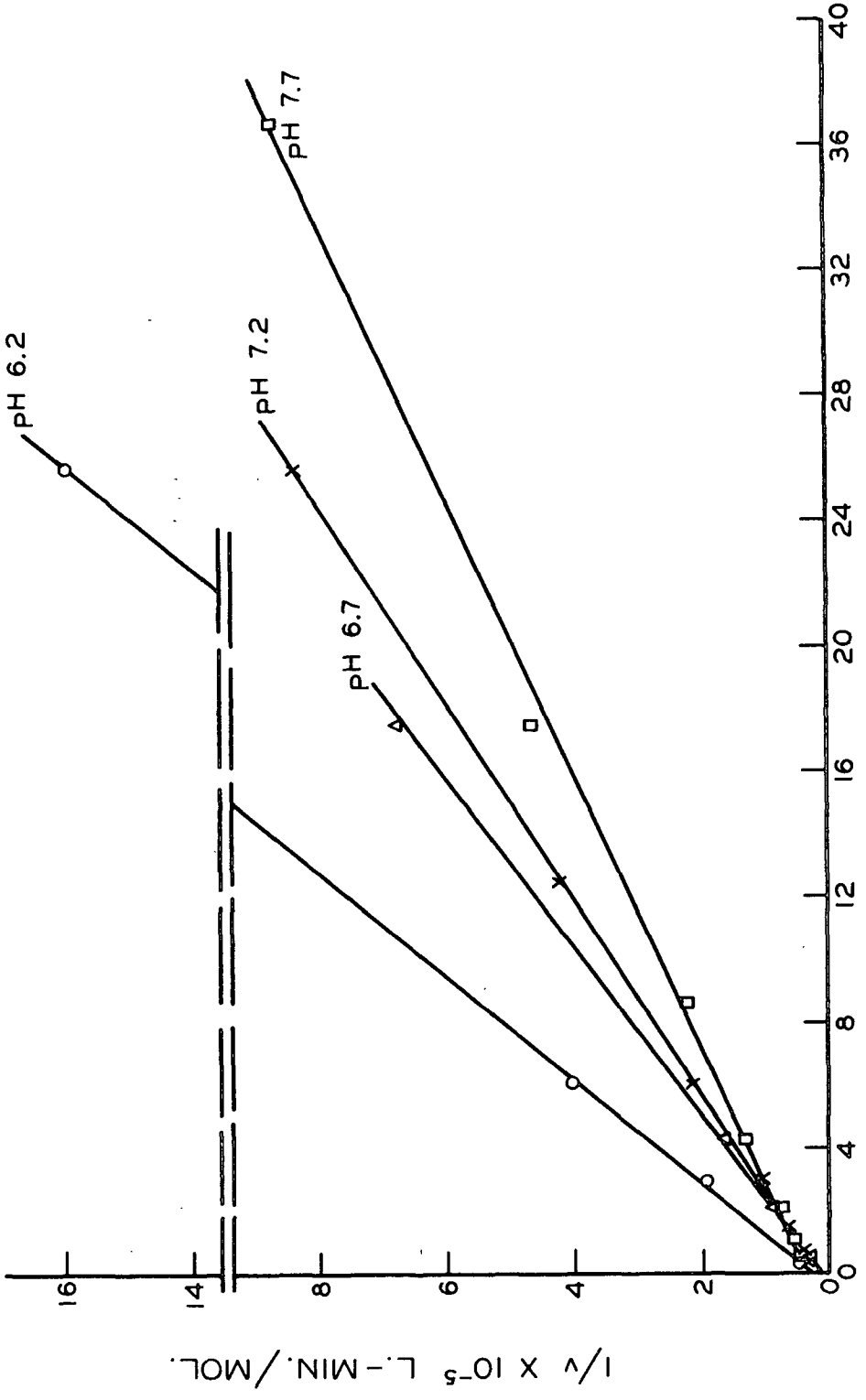

Figure 22. Effect of pH on the Formation of Guaiacylglycerol-β-coniferyl Ether in the Coniferyl Alcohol-Pseudolaccase K System as Plotted by the Method of Lineweaver and Burk (64) (All Buffers Were 0.05M Phosphate; the Temperature Was 25°C.; Data Collected by C^{14} Counting)

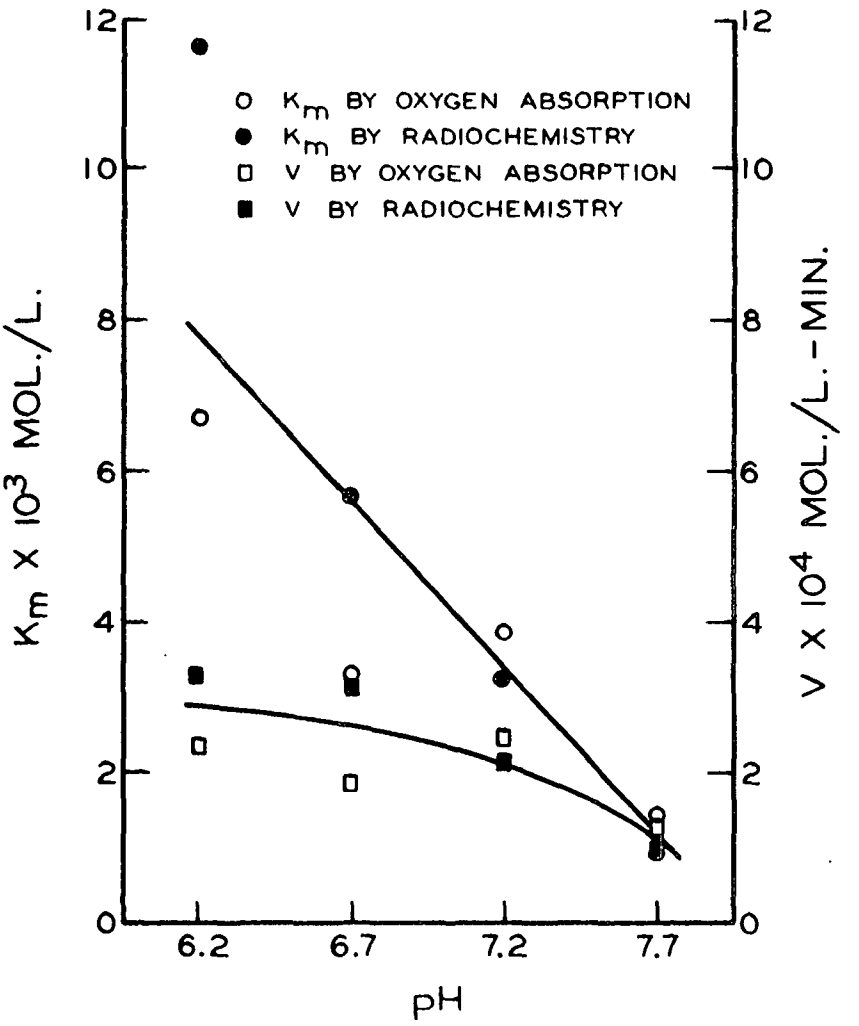

Figure 23. Effect of pH on the Maximum Velocity, V, and Michaelis Constant, K_m, for the Coniferyl Alcohol-Pseudolaccase K System in ~0.05M Phosphate Buffer at 25°C.

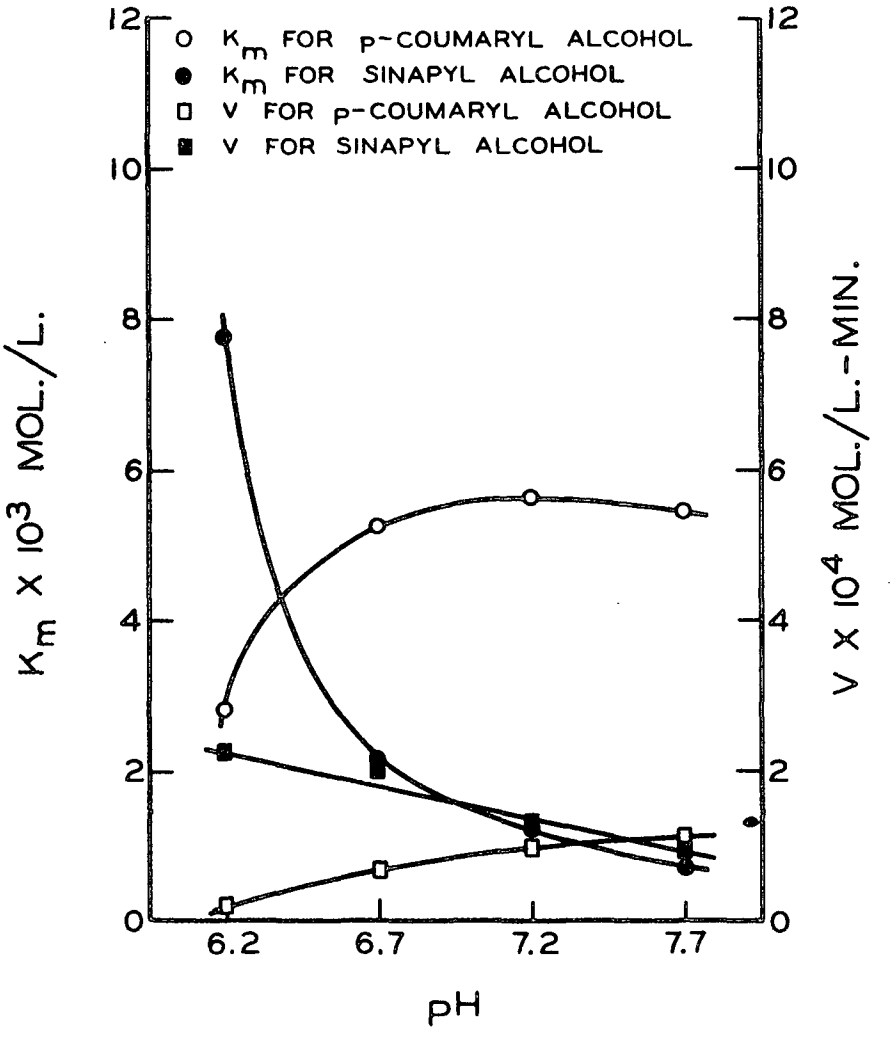

Figure 24. Effect of pH on the Maximum Velocity, V, and Michaelis Constant, K_m, for the p-Coumaryl- and Sinapyl Alcohol-Pseudolaccase-K Systems in 0.05M Phosphate Buffer at 25°C. (Oxygen Absorption Data)

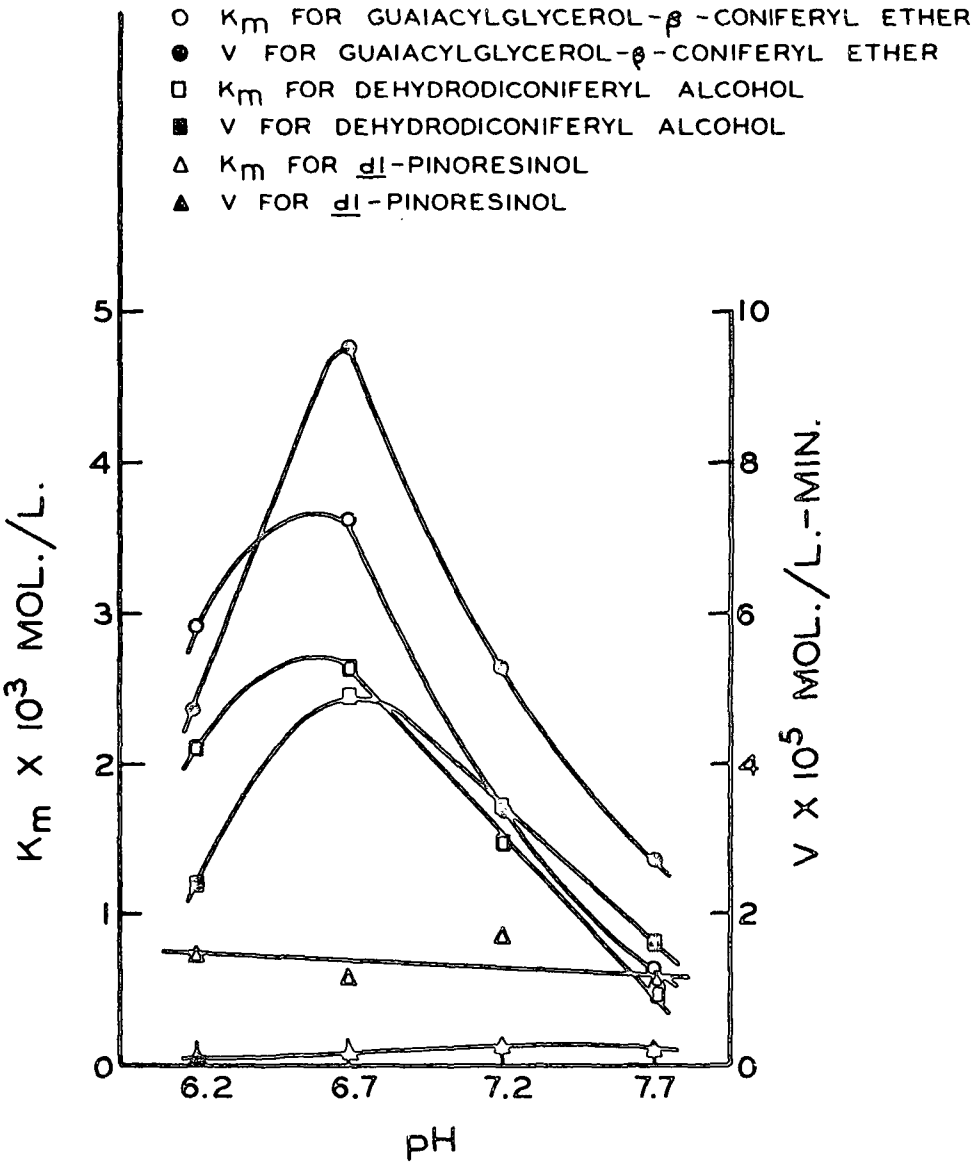

Figure 25. Effect of pH on the Maximum Velocity, V, and the Michaelis Constant, K_m, for the Principal Dimers Originating in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer at 25°C. (Data by Radiochemistry)

TABLE VIII

REACTION RATE DATA

Substrate or Product	pH	Lineweaver-Burk Data: Slope	Lineweaver-Burk Data: Intercept x 10^{-4}	V x 10^{4} moles/liter-minute	K_m x 10^{3} moles/liter
p-Coumaryl alcohol[a]	6.2	165.96	5.9159	0.1690	2.802
	6.7	76.898	1.4605	0.6850	5.265
	7.2	57.705	1.0298	0.9718	5.610
	7.7	49.688	0.9171	1.0910	5.420
Sinapyl alcohol[a]	6.2	34.726	0.4490	2.2270	7.740
	6.7	11.075	0.5079	1.9680	2.179
	7.2	9.533	0.7780	1.2870	1.226
	7.7	7.645	1.0906	0.9065	0.693
Coniferyl alcohol[a]	6.2	28.767	0.4301	2.3230	6.690
	6.7	17.783	0.5409	1.8500	3.305
	7.2	15.982	0.4133	2.4200	3.865
	7.7	10.998	0.7772	1.2870	1.414
Coniferyl alcohol[b]	6.2	35.578	0.3063	3.2640	11.610
	6.7	18.270	0.3237	3.0950	5.650
	7.2	15.248	0.4736	2.1150	3.222
	7.7	9.908	1.0256	0.9760	0.967
Dehydrodiconiferyl alcohol[b]	6.2	87.500	4.150	0.2410	2.108
	6.7	54.030	2.040	0.4901	2.645
	7.2	45.290	3.095	0.3235	1.465
	7.7	28.830	6.197	0.1615	0.466
dl-Pinoresinol[b]	6.2	742.20	99.80	0.0100	0.744
	6.7	371.10	62.45	0.0160	0.595
	7.2	343.40	40.03	0.0250	0.858
	7.7	262.90	45.45	0.0220	0.579
Guaiacylglycerol-β-coniferyl ether[b]	6.2	61.220	2.112	0.4740	2.900
	6.7	37.810	1.048	0.9550	3.613
	7.2	32.130	1.898	0.5274	1.695
	7.7	23.030	3.713	0.2697	0.622

[a] Oxygen absorption data.

[b] Radiochemistry data.

was supported. All intercepts and slopes were calculated by the least-squares method of straight-line data analysis. Correlation coefficients were greater than 0.993.

The pH optimum for the disappearance of coniferyl and sinapyl alcohols laid outside the pH range investigated on the acid side. For p-coumaryl alcohol, the pH optimum for substrate disappearance was outside the pH range tested on the alkaline side. Dehydrodiconiferyl alcohol and guaiacylglycerol-β-coniferyl ether were formed most rapidly at pH 6.7 while pinoresinol was produced most rapidly at pH 7.5.

The presence of dimers in the solution of a β-C^{14}-coniferyl alcohol-pseudolaccase K system as a percentage of the coniferyl alcohol reacted is plotted vs. time in Fig. 26. Dehydrodiconiferyl alcohol accounted for about 35% of the coniferyl alcohol reacted at 2 and 5 minutes. Substrate concentration and pH showed no measurable influence on the amount of dehydrodiconiferyl alcohol present after 2 and 5 minutes reaction time; but at longer times, 10 and 15 minutes, the percentage of dehydrodiconiferyl alcohol decreased with decreasing pH. The dehydrodiconiferyl alcohol present at 15 minutes as a percentage of the coniferyl alcohol reacted was 32.1, 32.2, 32.7, and 32.8 at pH 6.2, 6.7, 7.2, and 7.7, respectively. Substrate concentration appeared to have no influence on the dehydrodiconiferyl alcohol present in the solution at 10 and 15 minutes.

Guaiacylglycerol-β-coniferyl ether accounted for about 50% of the coniferyl alcohol reacted at 2 minutes. The actual values appeared to be dependent on pH and on substrate concentration at pH 7.2 and 7.7.

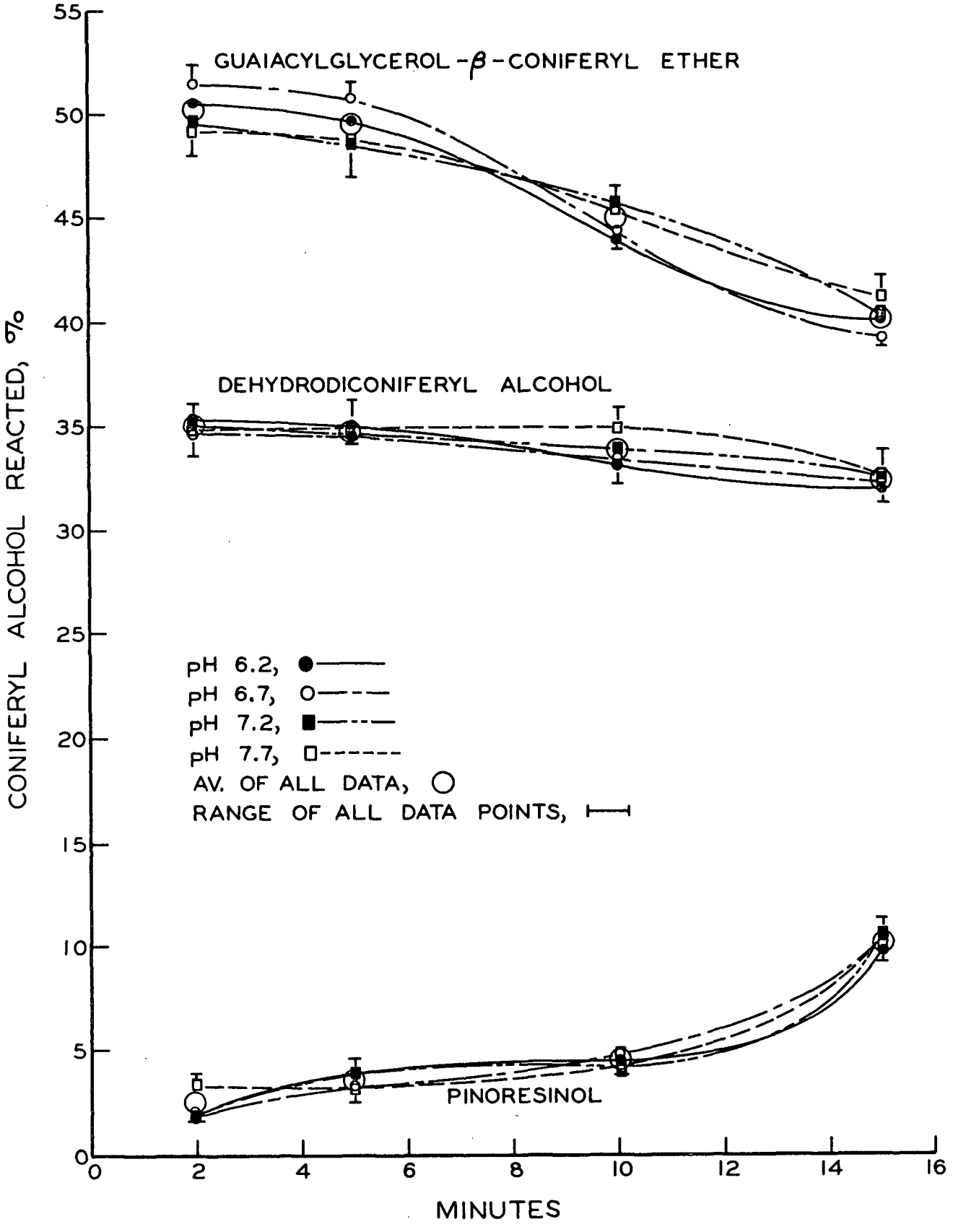

Figure 26. The Presence of the Principal Dimers in a Solution of the β-C^{14}-Coniferyl Alcohol-Pseudolaccase K System as a Percentage of the Coniferyl Alcohol Reacted

The percentage increased with decreasing substrate concentration at pH 7.2 and 7.7 at 2 and 5 minutes only. Substrate concentration showed no influence on the guaiacylglycerol-β-coniferyl ether at other times and pHs. The percentages of guaiacylglycerol-β-coniferyl ether present based on the coniferyl alcohol reacted were 51.5, 50.6, 49.7, and 49.2 at pH 6.7, 6.2, 7.2, and 7.7, respectively, at 2 minutes. At 15 minutes, the percentages were 39.3, 40.2, 40.5, and 41.5 at pH 6.7, 6.2, 7.2, and 7.7, respectively. It was noted that the trends in the percentages as a function of pH were opposite at 2 and 15 minutes.

Pinoresinol accounted for about 2.5% of the coniferyl alcohol reacted at 2 minutes. At 5, 10, and 15 minutes, pinoresinol accounted for 3.5, 4.4, and 10.2%, respectively. Substrate concentration and pH appeared to have no influence on the pinoresinol present in solution at any time on the basis of the coniferyl alcohol reacted (Table IX).

TABLE IX

PRINCIPAL DIMERS IN A SOLUTION OF THE β-C^{14}-CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM AS A PERCENTAGE OF THE CONIFERYL ALCOHOL REACTED

(Data collected at 2, 5, 10, and 15 minutes)

pH	Dehydrodiconiferyl Alcohol, %				Guaiacylglycerol-β-coniferyl Ether, %				Pinoresinol, %			
	2	5	10	15	2	5	10	15	2	5	10	15
6.2	35.3	35.1	33.2	32.1	50.6	49.7	44.1	40.2	2.04	3.95	4.43	9.8
6.7	34.8	34.7	33.6	32.2	51.5	50.9	44.4	39.3	1.93	3.17	4.66	10.2
7.2	35.0	35.0	34.1	32.7	49.7	48.6	45.9	40.5	1.97	3.94	4.19	10.4
7.7	34.9	34.9	35.0	32.8	49.2	48.8	45.6	41.4	3.55	3.16	4.22	10.3
Av.	35.0	34.9	34.0	32.4	50.2	49.5	45.0	40.3	2.37	3.56	4.38	10.2

PAPER CHROMATOGRAPHY

The chromatographic behavior of the reaction products of the coniferyl alcohol-pseudolaccase K system was compared with that of authentic samples supplied by Freudenberg. Immediately following oxygen absorption data collection, portions of the reaction substrate-enzyme systems were spotted on Whatman No. 1 chromatographic paper which was previously soaked in ethyl acetate-formamide (5:1) and air dried. The chromatograms were developed with descending XMF developer for 8 or 9 hours. The results were excellent when the developing tank was freshly cleaned, dried with respect to water vapor, and saturated with developer vapors. The sinapyl- and p-coumaryl alcohol-pseudolaccase K systems were similarly characterized.

The above procedure was repeated many times for the radiochemistry data collection and average R_{ca} values for the products of coniferyl alcohol oxidation appear in Table X, which contains a summary of all results.

TABLE X

RESULTS OF PAPER CHROMATOGRAPHY

(Developer in all cases was XMF)

Authentic Material[a] or Substrate Alcohol	R_{ca}[b]	Color with PNA + Sat. Na_2CO_3	Color with Wiesner Test[c]	Literature Values[d]
Dehydrodiconiferyl alcohol (A)[a]	0.41	Purple	None	0.46
dl-Pinoresinol (B)[a]	1.51	Purple	"	1.79
Mixture (A+B+C[e]+ 4 unknowns)[a]	0.012	Purple	"	
	0.060	Purple	"	
	0.15 (C)	Violet	"	0.167
	0.195	Purple	"	
	0.27	Gray-purple	Rose	(0.37)[f]
	0.41 (A)	Purple	None	0.46
	1.51 (B)	Purple	"	1.79
Coniferyl alcohol	0.012	Purple	None	
	0.059	Purple	"	
	0.15	Violet	"	
	0.20	Purple	"	
	0.28	Gray-purple	Rose	
	0.41	Purple	None	
	1.00	Robin's egg blue	Rose (light)[g]	
	1.24	None	Rose	(1.33)[h]
	1.49	Purple	None	
	1.65	None	Rose	i
p-Coumaryl alcohol	0.095	Peach		
	0.39	Pink		
	1.00	Royal blue		
	1.85	Pink		
Sinapyl alcohol	0.056	Sky blue		
	0.135	Sky blue		
	0.32	Royal blue		
	0.36	Yellow-brown		
	0.43	Sky blue		
	1.00	Yellow		
	1.46	Yellow		
Syringaresinol[a]	1.30	Yellow		

[a] Authentic materials were obtained from K. Freudenberg.
[b] R_{ca}, displacement from the origin relative to the respective substrate.
[c] The Wiesner test is specific for cinnamaldehydes.
[d] The values of Freudenberg and Lehmann (18) reduced to the R_{ca} convention.
[e] C equals guaiacylglycerol-β-coniferyl ether.
[f] R_{ca} for the aldehyde of guaiacylglycerol-β-coniferyl ether (18).
[g] Positive aldehyde test due to auto-oxidation of coniferyl alcohol during overnight air-drying of the chromatogram.
[h] R_{ca} for the aldehyde of dehydrodiconiferyl alcohol (18).
[i] No literature value given but in proper position for coniferaldehyde.

DISCUSSION

PSEUDOLACCASE K

Preparation G_3-2 of this research was obtained by carefully following the methanol fractionation procedures of Freudenberg, et al. (12) with two exceptions which are believed to have been without influence on the fractionation efficiency. The brown color of the Freudenberg enzyme preparation was not duplicated in preparation G_3-2 because the ascorbic acid in the buffer solutions prevented the formation of brown melanin-like materials. Harkin (26) reported a mushroom enzyme preparation with a gold-yellow color; it was prepared according to Freudenberg, et al. (12). Much gold-yellow material was removed from preparation G_3-2 during desalting with Sephadex G-25 which removed materials up to ca. 3000 molecular weight (ca. 70% of G_3-2). Freudenberg, et al. (12) reported a light brown solution of "mushroom laccase" which was free of tyrosinase, peroxidase, and catalase, and possessed traces of a β-glucosidase. Analytical electrophoresis of the desalted preparation H_3-2 revealed five components, all of which were enzymatically active (see Fig. 4, page 24). Experiments indicated that component I was tyrosinase; it accounted for ca. 10 to 15% of preparation H_3-2. Freudenberg, et al. (12) reported that inactivation of their enzyme occurred during the dehydrogenation of catechol. Such inactivation is a property typical of tyrosinase (33). Preparation H_3-2 behaved similarly; but pseudolaccase K, preparation H_3-2 with tyrosinase and component V removed and components II and IV partially removed by zone electrophoresis, was not inactivated during the dehydrogenative oxidation of catechol. Carbon monoxide inhibition of pseudolaccase K was negligible, but components I and V of preparation H_3-2 were strongly inactivated.

The pH range of uninhibited activity of component I (pH 5.4 to 8.0) agreed well with literature values for tyrosinase (33). On the basis of biological activity measurements, Higuchi (72) concluded that the Freudenberg enzyme preparation was a mixture of tyrosinase and "laccase." The present research has revealed unambiguously the multicomponent nature of a preparation similar to the Freudenberg enzyme preparation, no component of which possessed the physical, chemical, and biological activity properties of lacquer tree laccase (43,72).

Fåhraeus and Ljunggren (73) studied the substrate specificity of a fungal laccase which was obtained as an exoenzyme from a 2,5-xylidine induced fungus (Polyporous versicolor). For comparison purposes, they mistakenly considered that the "mushroom laccase" of Freudenberg, et al. (12) was a pure preparation. They did, however, cite the possibility that their laccase and the Freudenberg "laccase" might be different physical entities.

The pseudolaccase K fractionation (Fig. 15, page 46) showed that the three components of pseudolaccase K catalyzed the oxidation of hydroquinone, ferulic acid, and coniferyl alcohol. Components III and IV also catalyzed the oxidation of syringaldehyde. Ascorbic acid was oxidized at a relatively low rate but with equal facility by all three components. The fact that the oxidation of vanillin was not catalyzed by any fraction of pseudolaccase K revealed the greatest difference between the biological activity properties of pseudolaccase K and lacquer tree laccase. The latter exhibited a moderate ability to catalyze the oxidation of vanillin (72). The specific activities of component III

were measured by the fraction possessing the largest Q_{O_2} with respect to various substrates. Comparison of these specific activities or the oxygen consumption-time curves from which they were calculated with specific activities or oxygen absorption-time curves reported for laccase (72,73) indicated that pseudolaccase K and laccase possessed quantitative differences in biological activity as well as marked differences in chemical and physical properties.

It was difficult to estimate the weight ratios of the three components in pseudolaccase K from Fig. 15, but component III was, relatively, highly active. The most active fraction of pseudolaccase K possessed an $Q_{O_2\text{-}HQ}$ (25°C., pH 6.8, 0.05M phosphate buffer) equal to 77,000 which was nearly four times more active than the best preparation of Freudenberg, et al. (12). It was twice as active as the preparation of Fåhraeus, et al. (41). Therefore, component III is the activity to which the name pseudolaccase should specifically apply if, in the future, pseudolaccase K is separated into its individual components.

Analytical electrophoresis and ultracentrifuge studies have long been used to investigate the homogeneity of protein and enzyme preparations. Alberty (56,57) and others (58-61) showed that a single peak on a schlieren electrophoresis pattern was not sufficient evidence to call a preparation homogeneous. They refined analytical electrophoresis to yield a heterogeneity coefficient as an index of the electrophoretic inhomogeneity of proteins and enzymes. They showed that 12 of 14 "pure" proteins and enzymes (7 were crystalline preparations) possessed electrophoretic mobility distributions. While electrophoresis spreading experiments indicated a substantial mobility distribution for pseudolaccase K,

the method did not reveal the multicomponent nature of pseudolaccase K (see Fig. 8, pages 32 and 33; text, page 31). The sedimentation equilibrium experiment revealed a distribution of molecular weights, but the three-component nature of pseudolaccase K was revealed only by the zone electrophoresis fraction characterization experiment.

The sequence of results cited above exemplifies the necessity of employing all possible methods of physical characterization of enzyme preparations before positive statements as to purity are made. The heterogeneity coefficients reported in this research for pseudolaccase K are characteristic only of this preparation. Future electrophoresis spreading experiments on the individual pseudolaccase K components, II, III, and IV, would be expected to yield new values of β. The results of the present work indicate that each component will possess an electrophoretic mobility distribution large enough to measure.

The copper content of the fractions of pseudolaccase K averaged 0.188% with no correlation of copper content and nitrogen content or biological activity. This was inconsistent with the behavior of laccase from the Indo-Chinese lacquer tree (R. succedanea) for which Keilen and Mann (36) correlated copper content and specific activity for preparations of various purity. Freudenberg, et al. (12) reported that their "laccase" possessed 0.30% copper and its activity was enhanced upon addition of copper salts. This fact indicates the presence of tyrosinase or its apoenzyme in the Freudenberg enzyme preparation; recently, it was found (74) that copper salts activated the apoenzyme of tyrosinase which was found in crude preparations containing tyrosinase. The activity of pseudolaccase K was not increased upon addition of copper salts.

Malmstrom, et al. (75) used electron spin resonance studies to show that the copper of the laccase from P. versicolor was reduced during substrate oxidation. (Two forms of copper-enzyme bonding were indicated.) The blue enzyme preparation also became colorless upon reduction. It regained its blue color upon aeration. Nakamura (43) showed spectrophotometrically that laccase from the Chinese lacquer tree became leuco upon substrate oxidation and regained its blue color when aerated or oxidized with 0.1M ferricyanide solution. The intensity of the absorption maximum at 615 mμ was measured. Pseudolaccase K possessed no blue color or absorption maximum at 615 mμ (see Fig. 14, page 43). The results did not support, and tended to refute, the concept that pseudolaccase K contained oxidases in which copper was a necessary element of the active catalytic site. The copper content found may have been due to random adsorption of copper by the enzyme during processing or during the growth of the mushroom. Other metal ions, i.e., manganese or iron, may be located at the active site, or the active site may be devoid of metal ions. The copper content may be required for oxidation of the reduced enzyme by a reaction which was not rate controlling. Fundamental investigations into this unresolved question are needed to clarify the limited findings of this research. Oddly enough, 0.188% copper corresponded to one copper atom per enzyme molecule based on an average molecular weight of 35,000. However, a range of molecular weights were present; therefore, it was difficult to anticipate the stoichiometric relationships that actually existed. One or no copper atoms per molecule would, indeed, explain why carbon monoxide did not inactivate pseudolaccase K.

All laccases except the laccase of Fåhraeus, et al. (41) have possessed a carbohydrate component (positive Molisch reaction) which was large (ca. 50% of the enzyme) when it was assumed that the non-protein part of the enzyme was all carbohydrate. The laccase of Fåhraeus, et al. possessed less than 1% carbohydrate by actual measurement. By difference, based on the nitrogen content and assuming the protein contained 16% nitrogen, pseudolaccase K was about two-thirds nonproteinaceous. Qualitative chromatographic analysis of a pseudolaccase K hydrolyzate produced 9 amino-acid spots and 2 sugar spots. Galactose and xylose in a ratio of about 10:1 appeared as spots (after spraying with p-A) which were not intense enough to account for two-thirds of the material spotted on the chromatogram. It is suggested that part of the enzyme in addition to protein and carbohydrate may be a cofactor or lipid.

The partial specific volume of proteins usually lies between 0.70 and 0.75 ml./g. (52) while that of carbohydrates lies between 0.60 and 0.65 ml./g. (76). The partial specific volume of pseudolaccase K was 0.663 ml./g. which was in good agreement for an enzyme comprised of both protein and carbohydrate. Nakamura (43) calculated a molecular weight of 120,000 for Chinese lacquer tree laccase based on an assumed partial specific volume of 0.75 ml./g. The laccase was 9% nitrogen and, by difference, 45% carbohydrate; thus, the calculated molecular weight of 120,000 was probably several per cent too high.

REACTION RATE STUDIES

A contribution of this research was the establishment of rate constants for the p-coumaryl, sinapyl, and coniferyl alcohol-pseudolaccase

K systems as a function of pH in the physiological range. These rate studies serve two major purposes. First, the measurements of rates are the principal means for the description of enzyme-catalyzed reactions, and thus provide the main tool in the discovery, purification, and characterization of enzymes. In addition, the quantitative study of the kinetics of enzyme action has led to important conclusions about the manner in which enzymes act as catalysts in biochemical reactions. Of particular significance have been studies of the kinetics of enzyme-catalyzed reactions as a function of initial substrate concentration and of temperature and pH. Secondly, the substrate alcohols studied have been suggested as the monomeric precursors of coniferous and deciduous mono- and dicotyledenous lignins.

Reaction rate studies were conducted in a manner consistent with the Michaelis-Menten (65) theory of an enzyme-substrate complex[a]. See Equation (5). The theory was based on the assumption that the enzyme

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (5)$$

formed a reversibly dissociable complex with the substrate. Instantaneous equilibrium of such complex formation was presumed. First-order reaction rate theory dealt with those cases in which only one molecule of substrate combined with each independent enzymatic site of catalysis. The thermodynamic activities of the substances in the reaction mixture were assumed

[a]The studies of Chance (77) have validated the use of the Michaelis-Menten theory. By means of direct spectroscopic measurements, Chance studied intermediate substrate-enzyme complexes, determined the sequence of their formation and disappearance, and showed which complex was rate controlling for a particular set of experimental conditions.

equal to their respective concentrations. Reaction rate data taken from systems with substrate concentrations low enough to maintain unoccupied enzymatic sites at all times (see Appendix VII) were easily analyzed when employing a Lineweaver-Burk (64) plot.

The data for p-coumaryl, sinapyl, and coniferyl alcohols were in excellent agreement with first-order reaction rate theory and the Michaelis-Menten concept of an enzyme-substrate complex at the hydrogen ion and substrate concentrations employed. While this does not completely define the reaction mechanism, higher order rate theories need not be considered. The data did not reveal the nature of the product as defined in Equation (5). Since the formation of the principal dimers in the coniferyl alcohol-pseudolaccase K system also followed first-order kinetics, the conversion of possible nondimeric products to dimers was not rate controlling. Freudenberg, et al. (12,16,24,26) have hypothesized a free radical as the product defined in Equation (5). The data of this research did not directly refute this hypothesis because free radical reactions, producing dimers in this case (see Fig. 1, page 3), are acknowledged to be fast and, therefore, not rate controlling.

Assuming that pseudolaccase K and laccase were reoxidized in analogous ways following their reduction with substrate oxidation, the possible rate-controlling reoxidation of enzyme was ruled out by the spectrophotometric and magnetometric studies of Nakamura (78). It was found that the oxidation of laccase by molecular oxygen proceeded very quickly, and that the value obtained for the second-order velocity constant was comparable to that of the reaction for the formation of the enzyme-substrate complex between horseradish peroxidase and hydrogen peroxide (79),

which means a high affinity of laccase copper for oxygen. It was also found that only the reduction of laccase by the substrate was pH dependent with the pH of optimum reaction at 7.4.

The initial reaction rate data collected by two independent methods, oxygen absorption [Equation (3), page 14] and direct measurement of substrate concentrations by a radiotracer method, were in good agreement. See Table XI. The stoichiometric relationship in Equation (3) was correct and valid for early reaction times. Harkin (26) found evidence of the nonoxidative disappearance of coniferyl alcohol at relatively long reaction times. The proportion of coniferyl alcohol utilized in addition reactions was inversely proportional to the enzyme concentration. The same behavior was noted in this research as can be seen by comparing Fig. 30 to 33 and 34 to 37 in Appendix IX. The decrease in dehydrodiconiferyl alcohol as a percentage of the coniferyl alcohol reacted with time (Fig. 26, page 61) was probably due to the addition of dehydrodiconiferyl alcohol to the quinone methide demonstrated by Harkin (26) (see Fig. 1, lower left, page 3), or the oxidation of dehydrodiconiferyl alcohol to the aldehyde of dehydrodiconiferyl alcohol (18) or both. The decrease in guaiacylglycerol-β-coniferyl ether as a percentage of the coniferyl alcohol reacted with time was probably due to the addition of coniferyl alcohol and dehydrodiconiferyl alcohol to the quinone methide mentioned above or its oxidation to the aldehyde of guaiacylglycerol-β-coniferyl ether (18) or both. Each alcohol addition precluded water addition which would have formed guaiacylglycerol-β-coniferyl ether.

TABLE XI

INITIAL REACTION RATE DATA FOR THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER SOLUTION AT 25°C.

pH	Substrate Concentration, $\bar{s}$, moles/liter x 10^3	Initial Rate Data Coniferyl Alcohol Consumed, moles/liter-minute x 10^5	
		Oxygen Absorption	Radiotracer
6.2	11.67	15.00	
	5.72	9.80	9.10
	2.80	7.52	
	1.37	4.08	
	0.67	1.96	2.08
	0.33	1.14	1.03
	0.16	0.54	
	0.078		0.22
6.7	16.67	16.32	
	8.17	12.56	
	4.00	10.92	11.1
	1.96	7.68	
	0.97	3.92	4.5
	0.47	2.27	2.48
	0.23	1.21	
	0.11	0.62	0.61
7.2	11.67	14.8	
	5.72	13.6	13.9
	2.80	12.8	10.5
	1.37	6.24	6.2
	0.67	3.60	3.68
	0.33	1.89	1.92
	0.16	0.96	1.00
	0.078		0.50
7.7	16.67	12.04	
	8.17	11.44	
	4.00	9.28	9.22
	1.96	7.64	7.75
	0.97	5.28	5.30
	0.47	3.16	3.10
	0.23	1.80	1.81
	0.11	0.96	0.97
	0.054		0.53

The following discussion is based on radiotracer data collected from the highest initial coniferyl alcohol concentration at each of the 4 pHs. At 10 and 15 minutes' reaction time, the decrease in guaiacylglycerol-β-coniferyl ether as a percentage of the coniferyl alcohol reacted accounted for ca. 40% of the coniferyl alcohol which reacted without dehydrogenation plus the dehydrodiconiferyl alcohol which disappeared. At 2 minutes' reaction time, ca. 12% of the total coniferyl alcohol reacted was unaccounted for by the principal dimers. At 10 and 15 minutes' reaction time, ca. 17% of the total coniferyl alcohol reacted, including ca. 9% of the dehydrogenated coniferyl alcohol, was not accounted for by the principal dimers. By difference, ca. 8% of the total coniferyl alcohol reacted must have done so by addition. Actual calculation also revealed that ca. 8% of the total coniferyl alcohol reacted, did so through addition reactions, which were unaccountable because trimers, tetramers, etc. were formed. The demonstration of the formation of 37 compounds other than the 3 principal dimers (18) made the speculation of the exact whereabouts of the unaccountable coniferyl alcohol a difficult task.

K_m, the Michaelis constant, equals $(k_2 + k_3)/k_1$ where k_1, k_2, and k_3 are defined by Equation (5). (See also Appendix VII.) The K_m calculated from experimental data equals the true dissociation constant, (k_2/k_1), only when k_2 is much greater than k_3. In most enzyme-catalyzed reactions, k_3 is sufficiently large to affect the equilibrium, $E + S \rightleftharpoons ES$; and unless it has been shown experimentally that k_2 is much greater than k_3, the reciprocal of K_m is not a measure of the association between E and S,

that is, enzyme-substrate affinity. However, a comparison of K_m and k_3 values, where $k_3 = V/(E_t)$, yields a qualitative measure of the relative enzyme-substrate affinities under comparable experimental conditions (80), such as reactions on two or more substrates by the same enzyme system or for reactions at two or more pHs involving the same substrate and enzyme. For example, if $(K_m)_1 \leqq (K_m)_2$ and $(V)_1 \geqq (V)_2$, the corresponding enzyme-substrate affinities, (k_2/k_1), would be greater for S_1 than for S_2.

In this research, there were no significant differences in enzyme-substrate affinities for a particular substrate as a function of pH (Table XII). The same was true for the formation of dimers. Coniferyl and sinapyl alcohols exhibited no significant difference in substrate-enzyme affinity under comparable experimental conditions, but both showed significantly stronger enzyme-substrate affinity than did p-coumaryl alcohol for comparable environments. These results were in agreement with earlier findings (26); i.e., di- and trihydroxy or substituted hydroxy cinnamyl alcohols were catalytically oxidized about 4 times more rapidly than monohydroxy cinnamyl alcohols. The same was generally true for all phenols but the degree covered a wide range (26).

The velocity constant, k_3, was proportional to V and, therefore, trends in V as a function of pH were best suited as an index of the pH optima of the reactions. Coniferyl and sinapyl alcohols were most rapidly oxidized at pH 6.2, but the shape of the curve of V vs. pH (see Fig. 23 and 24, pages 56 and 57) indicated a reaction velocity maximum below pH 6.2. Fåhraeus and Ljunggren (73) found that guaiacol, catechol, and

TABLE XII

RELATIVE ENZYME-SUBSTRATE AFFINITIES

Substrate, product, or pH	$\frac{K_{m6.2}}{K_{m6.7}}$	$\frac{V_{6.2}}{V_{6.7}}$	$\frac{K_{m6.7}}{K_{m7.2}}$	$\frac{V_{6.7}}{V_{7.2}}$	$\frac{K_{m7.2}}{K_{m7.7}}$	$\frac{V_{7.2}}{V_{7.7}}$
Coniferyl alcohol[a]	2.02	1.26	0.86	0.76	2.73	1.87
Coniferyl alcohol[b]	2.06	1.05	1.75	1.46	3.33	2.18
p-Coumaryl alcohol[a]	0.53	0.25	0.93	0.71	1.04	0.89
Sinapyl alcohol[a]	3.55	1.13	1.78	1.53	1.77	1.42
Dehydrodiconiferyl alcohol[b]	0.77	0.49	1.80	1.53	3.14	2.00
Guaiacylglycerol-β-coniferyl ether[b]	0.80	0.50	2.13	1.81	2.72	1.95
dl-Pinoresinol[b]	1.25	0.63	0.69	0.64	1.48	1.13
	$\frac{K_{mCA}}{K_{mpC}}$[c,e]	$\frac{V_{CA}}{V_{pC}}$	$\frac{K_{mCA}}{K_{mSA}}$	$\frac{V_{CA}}{V_{SA}}$	$\frac{K_{mSA}}{K_{mpC}}$[d,e]	$\frac{V_{SA}}{V_{pC}}$
6.2[a]	3.26	16.3	1.18	1.24	3.76	13.2
6.7	0.85	3.6	2.05	1.25	0.41	2.88
7.2	0.63	2.34	2.9	1.76	0.22	1.32
7.7	0.22	1.05	1.72	1.25	0.13	0.83
	$\frac{K_{mDA}}{K_{mP}}$[f,h]	$\frac{V_{DA}}{V_{P}}$	$\frac{K_{mDA}}{K_{mGG}}$	$\frac{V_{DA}}{V_{GG}}$	$\frac{K_{mGG}}{K_{mP}}$[g,h]	$\frac{V_{GG}}{V_{P}}$
6.2[b]	2.84	24	0.73	0.51	3.84	47.
6.7	4.45	30.6	0.73	0.52	6.0	58.
7.2	1.71	12.9	0.86	0.61	2.0	21.2
7.7	0.81	7.3	0.75	0.60	1.07	12.3

[a] Oxygen absorption data.
[b] Radiotracer data.
[c] Coniferyl alcohol.
[d] Sinapyl alcohol.
[e] p-Coumaryl alcohol.
[f] Dehydrodiconiferyl alcohol.
[g] Guaiacylglycerol-β-coniferyl ether.
[h] dl-Pinoresinol.

hydroquinone were catalytically oxidized most rapidly by the laccase from P. versicolor at pH 4. However, Freudenberg and Hubner (15) indicated that coniferyl alcohol was oxidized to DHP by mushroom enzymes most rapidly at pH 8 on the basis of the time required for visible precipitate formation and the yield after three days. Their results for systems at pH 5 and 8 were: precipitate formation in about 5 to 8 hours and 5 to 10 minutes, respectively; and yields of filtered precipitate after 3 days at 10 and 75% of theory, respectively. These results were qualitative and subject to many unstudied variables in connection with DHP formation, such as the influence of pH on the solubility of the DHP and products preceding its formation and the influence of pH on the rate of various addition reactions which produce trimers, tetramers, etc., and other side reactions.

p-Coumaryl alcohol was most rapidly oxidized at pH 7.7 but the shape of the curve of V vs. pH (see Fig. 24) indicated that the reaction velocity maximum occurred at more alkaline conditions. The opposed results regarding the magnitude of the reaction velocity maxima and the pHs at which they occurred for the monohydroxy cinnamyl alcohol and the di- and trisubstituted hydroxy cinnamyl alcohols indicated either a strong influence by methoxyl substitution of the benzene ring on the reactions at one enzymatic site or possibly two different catalytically active sites on the enzyme or both. The multiple enzyme system employed provides other obvious possibilities. The question remains unresolved.

In the coniferyl alcohol-pseudolaccase K system, dehydrodiconiferyl alcohol and guaiacylglycerol-β-coniferyl ether were formed most rapidly

at about pH 6.7 while pinoresinol formation was maximal at about pH 7.2. It has been hypothesized (12,16,24,25) that the three dimers originate through the random collision of coniferyl alcohol free radicals with subsequent rearrangement of the compounds so formed. If so, pH optima uncharacteristic of free radical reactions were indicated. Hydrogen ion concentration could conceivably influence the relative reactivity of the sterically unhindered statistically predominant forms of a free radical-proton complex (81).

The reaction between a coniferyl alcohol free radical and an intact coniferyl alcohol molecule producing a dimeric free radical which is subsequently dehydrogenated to yield a quinone methide has been proposed (82). Such a reaction is not inconceivable, but the end result would be identical with that proposed by the Freudenberg school. Such a reaction does not explain the pH influence on the proposed free radical mechanism of dimer formation. Harkin (26) discredited the reaction between the coniferyl alcohol free radical and an intact coniferyl alcohol molecule, but the complete lack of fundamental studies of coniferyl alcohol chemistry makes the conclusion of Harkin questionable.

Adler (82) proposed two successive single electron transfer reactions resulting in coniferyl alcohol cations. See Fig. 27. It was suggested that copper was reduced during both electron transfer reactions. It is conceivable that reactions involving such cations could be susceptible to hydrogen ion concentration. The combination of cations would also lead to formation of the quinone methide observed by Harkin (26).

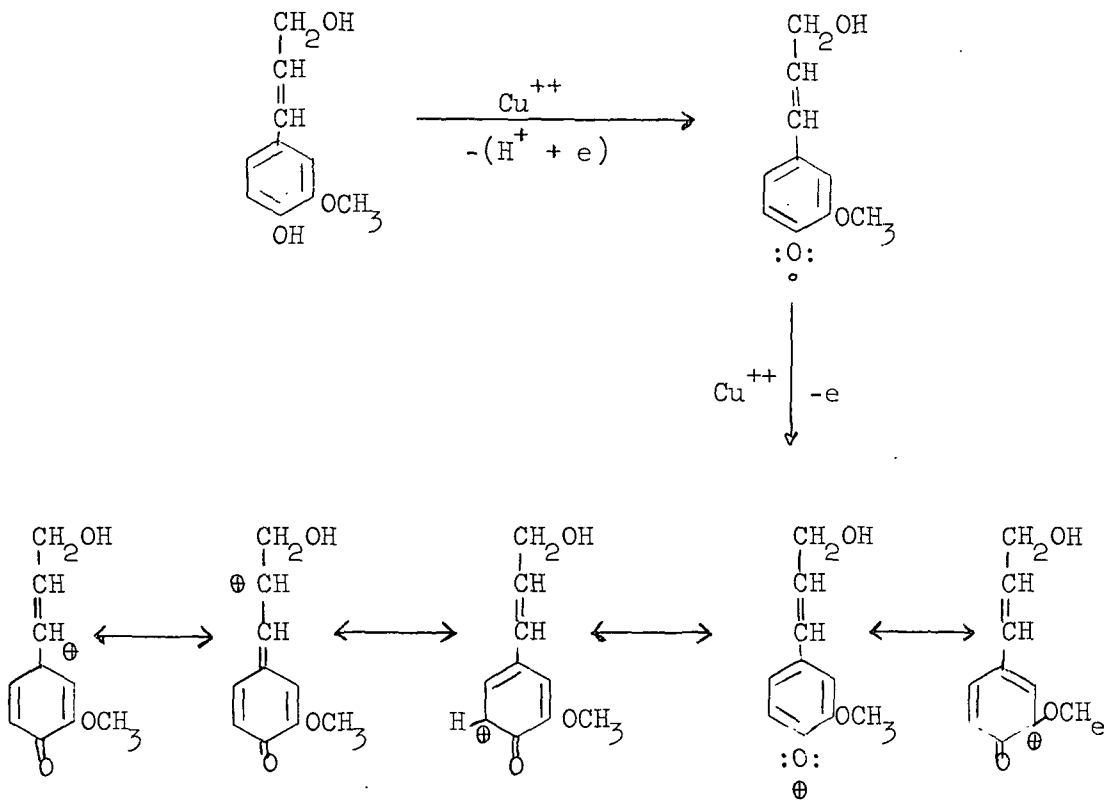

Figure 27. An Ionic Mechanism Capable of Dimer Formation According to Adler (82)

The data for the formation of pinoresinol (Fig. 26) indicated a strong activation of pinoresinol formation between 10 and 15 minutes' reaction time. The lag period prior to activation was inversely proportional to the initial concentration of coniferyl alcohol. A possible explanation was that some reaction product reached sufficiently high concentration after about 10 minutes to activate pinoresinol formation. Such a possibility appeared to be incompatible with the free-radical hypothesis.

Brauns and Brauns (83) reviewed the literature of the decade prior to 1959 on the biosynthesis of lignin and among their concluding remarks was the following:

> "dl-Pinoresinol (dl-P), when treated with oxygen in the presence of mushroom enzyme at pH 7, yielded a DHP-dl-P which did not give the lignin color reactions--in other words, it did not contain the carbonyl group responsible for these reactions. Furthermore, attempts to isolate bis(hydroxymethyl)-succinic acid dilactone--which is obtained in an optically active form from d-pinoresinol on oxidation with nitric acid--from DHP-dl-P or from lignin failed. This failure may indicate that the pinoresinol structure, if it occurs in the lignin molecule, is present in such a minute amount that it is difficult to detect, or that it has been changed to such an extent that it no longer gives the dilactone; in other words, that this structure is no longer present in the lignin molecule. Furthermore, it should be mentioned that nature produces an optically active d-pinoresinol in coniferous woods, and it is hard to believe that the plant produces an optically active and also an optically inactive pinoresinol, each with 4 asymmetric carbon atoms, and uses only the latter in the formation of lignin. In this respect, the enzymatic dehydrogenation of d-pinoresinol would have been of particular significance if the DHP formed had been tested for its optical activity and its ability to yield Hibbert's ketones. If the resulting DHP is optically active it would indicate that the activity of at least some of the asymmetric carbon atoms, probably the β-carbon atoms, had been preserved and that the carbon to carbon linkage between the β-carbon atoms had not been ruptured. If the DHP is optically inactive, then the whole bis-(hydroxymethyl)succinic acid dilactone system must have been destroyed and therefore could not have been incorporated into the polymer."

This thesis supports the view that only small amounts (2 or 3%) of pinoresinol may be incorporated into the DHP assuming the Freudenberg theory is correct. At low coniferyl alcohol concentrations, only 2 or 3% of the coniferyl alcohol reacted was measured as pinoresinol.

FUTURE RESEARCH

This author believes that fundamental investigations of the synthesis, degradation, and characterization of lignins and ligninlike polymers should

be attacked through the disciplines of enzymology and biochemistry. Research of the past decade would indicate that enzymes control the formation of lignins; the ligninlike DHP extensively studied by the Freudenberg school is synthesized by the enzymatically-catalyzed oxidation of coniferyl alcohol. Model compounds should be used to determine the ability of particular enzymes to make and break specific bond types characteristic of lignin and ligninlike polymers.

The isolation and characterization of pure pseudolaccase and cambial enzymes should be completed. Some specific problems are cited here with suggestions for obtaining their solutions.

Elution chromatography employing packed columns of Amberlite XE-64 ion-exchange resin (43,84) or diethylaminoethyl cellulose (DEAE-cellulose) or other cellulose derivatives (74,85) has exhibited high resolution in the separation of enzymes and proteins. Application of these techniques may separate the three-component pseudolaccase K and permit characterization of each component. Exceptional resolution could make purification by zone electrophoresis obsolete as it was applied in this research. The introduction of microphore[a] into the field of ionography demands critical evaluation as a preparative method.

The hypothesis that dimer formation proceeds via free radicals (12, 16,24,25) could be vigorously studied in a series of electron para-magnetic

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[a]Microphore is a microporous cellulose acetate paper-thin sheet made with uniform 3-micron diameter pores. It is manufactured by Gelman Instrument Company, 106 N. Main St., Chelsea, Michigan.

resonance (EPR) experiments. Similar experiments would also prove or disprove the existence of a metal ion as the electron transfer agent of the enzyme, identify the metal ion; e.g., copper, manganese, iron, etc., and reveal its valence states.

The pH influence on the kinetics of the addition reactions between quinone methides and primary alcohols, especially coniferyl and dehydrodiconiferyl alcohol, requires study. The solubility of DHPs and the secondary products preceding their formation should be investigated as a function of pH and buffer concentration.

SUMMARY AND CONCLUSIONS

The procedure of Freudenberg, et al. (12) was used to isolate "mushroom laccase" from the cultivated mushroom (Psalliota campestris). The "mushroom laccase" was a mixture of five enzymes. Three carbon monoxide-insensitive enzymes were isolated by zone electrophoresis and characterized as a group (pseudolaccase K), the most active component of which was named pseudolaccase. One of the two enzymes inactivated by carbon monoxide was tyrosinase. This research supported the conclusion of Higuchi (72) that the Freudenberg enzyme contained tyrosinase in addition to the enzymes responsible for the dehydrogenative oxidation of coniferyl alcohol. This thesis also supported the conclusion that, as performed, the methanol fractionation of the aqueous extracts of P. campestris failed to yield tyrosinase-free preparations.

The physical and chemical properties of pseudolaccase K from P. campestris and laccase from the Chinese lacquer tree (R. vernicifera) (43) and summarized in Table XIII. All properties differed significantly. The biological activities of pseudolaccase K and laccase were similar, but not identical, in their general ability to catalyze the oxidation of guaiacyl and syringyl compounds. The specific activity of pseudolaccase K equaled 35,300 with hydroquinone as substrate. This was 1.77 times as active as the "mushroom laccase" of Freudenberg, et al. and 4.4 times as active as the laccase characterized by Nakamura (43). The specific activity of the most active fraction of pseudolaccase K equaled 77,000 with hydroquinone as substrate. Purified pseudolaccase would be expected to possess a much higher activity. Pseudolaccase K

TABLE XIII

SUMMARY OF PSEUDOLACCASE K AND LACCASE PROPERTIES

Chemical or Physical Property	Pseudo-laccase K	Laccase[c]	Experimental Conditions pH	Buffer	Temperature, °C.	Remarks
Nitrogen content, %	3.94[a] 5.24[b]					Dumas method
		9.0				Kjeldahl method
Copper content, %	0.188					Bathocuproine method (71)
		0.22				Dithizone method
Protein content, %	32.8	55.0				Percentage of nitrogen x 6.25
Carbohydrate content, %	67.2[d]	45.0[d]	Molisch reaction (paper chromatography of pseudolaccase K hydrolyzate indicated galactose and xylose, ca. 10 to 1)			
Isoelectric pH	3.55	ca. 7.0				
Electrophoretic mobility, sq.cm./volt-second	0.65×10^{-4}		7	0.1M phosphate	0.5	Free electrophoresis
Heterogeneity coefficient, sq.cm./volt-sec.	1.02×10^{-5} 1.73×10^{-5} 1.08×10^{-5}		5 7 9	0.1M phthalate 0.1M phosphate 0.1μ veronal	0.5 0.5 0.5	" " "
Diffusion coefficient, sq.cm./sec.	7.9×10^{-7} 7.9×10^{-7} 12.5×10^{-7} 7.9×10^{-7}		5 7 9 7	0.1M phthalate 0.1M phosphate 0.1μ veronal 0.1M phosphate	0.5 0.5 0.5 20	" " " Ultracentrifuge
		4.63×10^{-7}			20	
Partial specific volume, ml./g.	0.663	(0.75)				Density gradient column Assumed
Sedimentation coefficient, s	3.82		7	0.1M phosphate	20	
		5.4	7	0.2μ phosphate	20	
Molecular weight	35,000	120,000				Sedimentation velocity
Absorption maximum, mμ	None	280 and 615				
Shoulder, mμ	280	330				
Color	Yellow	Deep blue				
Q_{O_2}-HQ	35,300		6.8	0.05M phosphate	25	
		8,000	7	0.033M phosphate	25	

[a] Preparation K.
[b] Weight average, Fractions 2 through 11 (see Fig. 15).
[c] Data by Nakamura (43)
[d] By difference.

was not blue and copper content did not correlate with specific activity; therefore, it was concluded that copper may not be the metal ion at the active site of pseudolaccase K components. In addition to protein comprised of at least 9 amino acids and carbohydrate made up of galactose and xylose (10:1), it was hypothesized that pseudolaccase K possessed lipid or a cofactor.

It was concluded that an enzyme possessing the physical, chemical, and precise biological activity properties of laccase did not exist in the methanol-fractionated aqueous extract of the cultivated mushroom (P. campestris). The results indicated that each of the three components of pseudolaccase K were able to catalyze the oxidation of coniferyl alcohol. The present research has revealed unambiguously the multi-component nature of a preparation similar to the Freudenberg enzyme preparation.

Sinapyl, coniferyl, and p-coumaryl alcohols were catalytically oxidized by pseudolaccase K according to first order kinetics; the Michaelis-Menten concept of an enzyme-substrate complex was supported at the experimental conditions employed. The formation of the principal dimers in the coniferyl alcohol-pseudolaccase K system satisfied the requirements of the Michaelis-Menten theory. The pH optimum of coniferyl- and sinapyl alcohol oxidation was below 6.2; that for p-coumaryl alcohol was above 7.7. It was concluded that, if but one type of enzymatic site was present in pseudolaccase K, methoxyl substitution ortho to the phenolic hydroxyl group of p-hydroxycinnamyl alcohol strongly influenced the pH optimum and magnitude of its activity; or independent sites were responsible for catalysis, one for the unsubstituted p-hydroxycinnamyl alcohol

and another for the methoxyl-substituted p-hydroxycinnamyl alcohols. Independent sites may lie on the same or different components of pseudolaccase K or both. The research of Fåhraeus and Ljunggren (73) on laccase suggested different catalytic sites for ortho-unsubstituted and ortho-substituted phenols.

Good agreement of initial reaction rate data from oxygen absorption and radiotracer experiments lead to the conclusion that the stoichiometric relationship between oxygen consumption and substrate dehydrogenation [Equation (3), page 14] was valid for the initial phases of the reaction yielding the principal dimers preceding DHP formation in the coniferyl alcohol-pseudolaccase K system for the conditions studied. Contrary to the opinion of Harkin (26), the Warburg constant volume respirometer proved to be a dependable instrument for obtaining initial reaction rate data from dilute (10^{-4} M) substrate systems.

At 2 and 15 minutes' reaction time, ca. 88 and 83%, respectively, of the coniferyl alcohol reacted was accounted for by the principal dimers. Addition reactions accounted for up to 8% of the coniferyl alcohol reacted after 10 minutes' reaction time. This is in complete agreement with the findings of Harkin (26) and his conclusion that 10 to 15% of the coniferyl alcohol in a DHP must be combined through addition to quinone methides. The percentage of coniferyl alcohol which reacted by addition, decreased as the relative concentration of enzyme increased. Relatively high concentrations of enzyme rapidly reduce the concentration of free coniferyl alcohol which is required for addition reactions.

In the coniferyl alcohol-pseudolaccase K system, dehydrodiconiferyl alcohol and guaiacylglycerol-β-coniferyl ether formation were maximal at pH 6.7; for pinoresinol, the pH of maximum formation was 7.2. Since free radical reactions are generally insensitive to moderate changes in hydrogen ion concentration, the results of this research indicated that a pH-sensitive mechanism was operating alone or in conjunction with the free radical mechanism of dimer formation. The fact that the pH dependence of dimer formation did not correlate with the pH dependence of coniferyl alcohol dehydrogenation supports the hypothesis that a nonfree radical mechanism operating alone, or in conjunction with the free radical mechanism, was responsible for dimer formation. The ionic mechanism hypothesized by Adler (82) deserves serious consideration. A second electron could be easily removed from the coniferyl alcohol free radical, creating a cation (carbonium ion), the mesomeric forms of which would be subject to equilibria influenced by hydrogen ion concentration. Combination of cations would yield the quinone methides previously detected by Harkin (26).

ACKNOWLEDGMENTS

I wish to express sincere appreciation to my Thesis Advisory Committee, Dr. I. A. Pearl, Dr. D. Williams, and especially Dr. E. O. Dillingham for their assistance and encouragement. I also wish to thank Dr. I. A. Pearl and Professor K. Freudenberg for donating authentic samples of compounds, Mr. Bernard Lueck of the Sulphite Pulp Manufacturers' Research League for instruction in the use of a Coleman Nitrogen Analyzer, and Mr. Lowell Sell for visible and ultraviolet spectroscopy.

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APPENDIX I

MUSHROOM STEM BASE EXTRACTION PROCEDURES

The fresh young mushroom stem bases were washed in deionized water to remove clinging dirt and manure. After cooling to 4°C., the washed stem bases were homogenized with 45% of their wet weight of buffer solution No. 1 in a chilled Waring Blendor. After approximately four minutes of homogenization, dry acid-washed sharp silica sand at -20°C. in an amount equal to one-half of the wet weight of the stem bases was added to the blendor and homogenization continued long enough to insure complete mixing. The temperature of the slurry rose to 10 to 12°C. The homogenate was immediately pressed through a nylon cloth by subjection to a pressure of approximately 400 pounds per square inch. (A hydraulic jack of 14 tons capacity was used to drive an eight-inch square hardwood platen into an appropriately designed hardwood box.) The mealy residue was stirred into an amount of buffer solution No. 1 equal to one-half the original wet weight of the stem bases and pressed. This rinse was repeated a second time. The aqueous extracts were combined and rapidly cooled to 0°C. by stirring in a stainless steel vessel placed in a deep freeze unit at -30°C. For a specific example, see the flow diagram in Appendix II, page 97.

APPENDIX II

FRACTIONATION PROCEDURES

Methanol at -30°C. was added to the vigorously stirred aqueous extract of mushroom stem bases at 0°C. over two hours until the total volume was 30% methanol. The undesired precipitate was centrifuged off as rapidly as possible. (All centrifugations were carried out batchwise at 1600 times gravity for fifteen minutes unless otherwise stated. A refrigerated continuous centrifuge would be desirable.) The precipitate was frozen, lyophilized, weighed, and set aside dry at -20°C. The decanted liquid was further cooled to -10°C. and additional methanol at -30°C. was added over two hours to the vigorously stirred solution until the total volume was 75% methanol. The light gray precipitate was immediately centrifuged from the golden yellow decantate and shaken in a polyethylene bottle with 800 ml. of buffer solution No. 2 for one hour at 0°C. Glass beads were added to aid solution of the precipitate (26). The suspension was centrifuged cold and the precipitate extracted two additional times with 400-ml. aliquots of buffer solution No. 2.

Cold methanol was added to the combined aqueous extracts as previously described until the total volume of the solution was 40% methanol. After centrifugation, the methanol content of the solution was increased to 75% as already described. The light gray precipitate was immediately centrifuged off and extracted with 100 ml. of buffer solution No. 2 for one hour at 0°C. The suspension was centrifuged and the precipitate extracted two additional times with 50-ml. portions of buffer solution No. 2. The combined extracts were stored overnight at 0°C. prior to desalting. The following flow diagram is a typical example of enzyme extraction and fractionation.

FLOW DIAGRAM I

MUSHROOM EXTRACTION AND EXTRACT PROCESSING

6.4 kg. of fresh mushroom stem bases (16 lots, each containing 400 g. of mushroom stem bases, 200 g. of acid-washed sharp silica sand, and 180 ml. of buffer solution No. 1) were homogenized for five minutes in a Waring Blendor and pressed through nylon cloth (pressure, about 400 lb./sq. in.). The temperature increased from 4°C. to 10 to 12°C. during homogenization.

↓

Press juice, 6.65 liters.

Press cake. Add 3.3 liters of buffer solution No. 1; mix and press

↓

Press juice, 3.3 liters.

Press cake. Add 3.3 liters of buffer solution No. 1; mix and press.

↓

Press juice, 3.3 liters.

Press cake. Discard.

↓

The juice from the three pressings was combined and cooled to 0°C.
Yield: 13.25 liters of slightly turbid, light tan solution A_3.
Solids: 36.4 mg./ml. (482 g.)
Q_{O_2}-HQ=43.1; Q_{O_2}-CAT=93.6; HQU-34,700; CATU=75,200

Add 5.7 liters of methanol at -30°C. to the aqueous extract over two hours. The final 30% methanol (v/v) solution at -4°C. was centrifuged immediately (1600 times gravity).

↓

Precipitate B_3 (145 g.); light muddy-tan colored.

Solution B_3
Yield: 18.75 liters; gold-yellow color.
Solids: 17.85 mg./ml. (335 g.)
Cool 17.6 liters of solution B_3 to -10°C. and add 31.7 liters of methanol at -30°C. over two hours. The final 70% methanol (v/v) solution at -20°C. was centrifuged immediately.

↓

Precipitate C_3 (47.8 g.); light gray color. Immediately add 800 ml. of buffer solution No. 2; shake for one hour and centrifuge.

Solution C_3
Yield: 47.37 liters; gold-yellow color
Solids: 5.6 mg./ml. (265 g.)

↓

Precipitate -- Immediately add 400 ml. of buffer solution No. 2; shake for one hour and centrifuge.

800 ml. of solution.

↓

Precipitate -- Immediately add 400 ml. of buffer solution No. 2; shake for one hour and centrifuge.

430 ml. of solution.

↓

Precipitate D_3 (10.5 g.)

420 ml. of solution.

↓

Combine the above solutions to give Solution D_3.
Yield: 1730 ml.
Solids: 21.5 mg./ml. (37.2 g.)
Q_{O_2}-HQ=193.5; Q_{O_2}-CAT=228; HQU=12,000; CATU=14,150

Cool solution D_3 to -2°C. and add 2.59 liters of methanol at -20°C. over two hours. The final 60% methanol (v/v) solution was centrifuged immediately.

↓

Precipitate E_3 (10.8 g.); light gray color. Immediately add 250 ml. of buffer solution No. 2; shake one hour and centrifuge.

Solution E_3; slight yellow color.
Yield: 4100 ml.
Solids: 6.45 mg./ml. (26.4 g.)
Add 2.62 liters of methanol at -26°C. over two hours. The final 75% methanol (v/v) solution was centrifuged immediately.

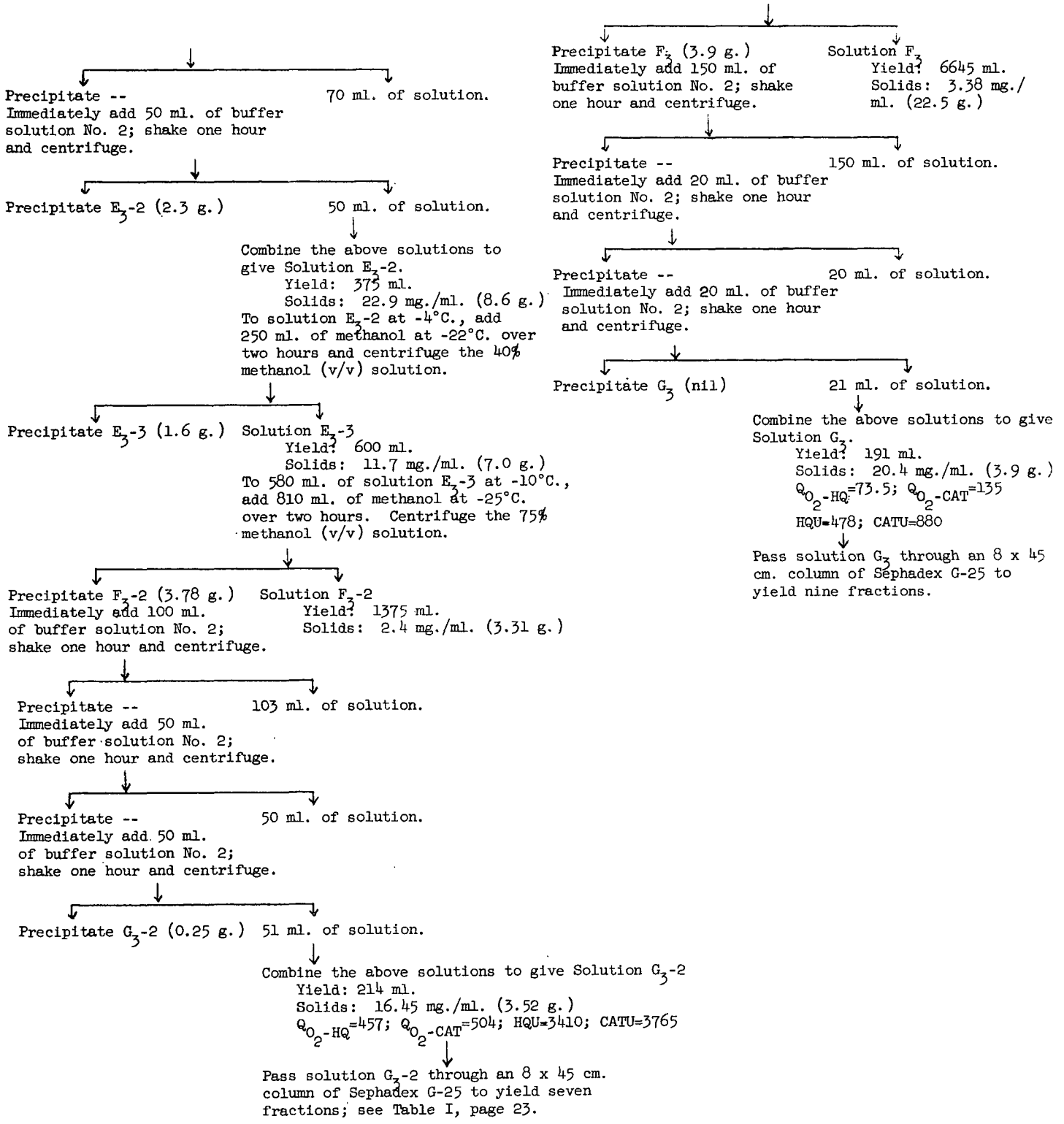
↓

Precipitate -- Immediately add 75 ml. of buffer solution No. 2; shake one hour and centrifuge.

255 ml. of solution.

↓

FLOW DIAGRAM I (continued)



APPENDIX III

PROCEDURES FOR PAPER ELECTROPHORESIS

Ionography is the term given to the preparative procedures used to obtain a record of the electrophoretic migration of charged particles, either ions or colloidally dispersed substances, through conducting solutions which have been stabilized with agar, gelatin, filter paper, starch, glass-fiber ribbon, or other materials (86). Whatman No. 1 filter paper saturated with 0.05M buffer solution of the desired pH was used in this work.

A saturated sheet of filter paper, 15 x 56 cm., was draped across the water-cooled stage of the migration chamber with each end hanging in a tank filled with the buffer solution used to saturate the sheet. Current was conveyed through a clip lead attached to a porous graphite electrode mounted in each tank. Approximately 5 λ of 0.1% enzyme solution were spotted on the paper, the chamber was closed, and 50 ma. of current at 400 volts were applied for 3 to 12 hours, depending on the migration rate.

After development, the filter paper-stabilized ionogram was partially dried in a horizontal position. (Complete drying from the liquid state inactivated the enzymes.) Appropriately cut strips of the ionogram were placed on 2% agar gel containing 0.25% chromophoric substrate. The distance from the origin to the center of the colored spot produced by the catalytic action of the enzyme on the chromophoric substrate (catechol or p-phenylenediamine) was measured, divided by the hours of ionogram development, and plotted as a function of pH on rectangular co-ordinates.

APPENDIX IV

ZONE ELECTROPHORESIS APPARATUS AND METHODS

The cell (Fig. 28) consisted simply of two glass cylinders of ca. 1.89 cm. internal diameter equipped with ball joints to accommodate the electrode vessels supplied with the Beckman/Spinco Model H Electrophoresis Instrument. The plain ends of the cylinders were sealed into a crystallization dish with a rubber stopper. It was readily apparent that the advantage of this cell was its 30 cm. uninterrupted vertical path for electrophoretic migration. When used for zone electrophoresis, the bottom cavity of the cell was filled with a solution of 1% agar and 25% sucrose in the desired buffer solution as solvent. (Buffer solution No. 4 performed best.)

After the agar solution gelled, the cell was assembled, filled with the desired cold buffer solution and placed in the constant temperature bath at about 1°C. A sucrose gradient (0 to 20% sucrose) was established in one leg of the cell by well-described methods (53-55). Two or three ml. of cold enzyme solution (1 to 5% enzyme and 20% sucrose in the desired buffer solution) were rapidly layered onto the agar surface at the base of the sucrose gradient. The layering was accomplished with a "syringe" consisting of a 50 cm. length of 3 mm. glass rod within a 45 cm. length of glass tubing of internal diameter slightly over 3 mm. A greased gum-rubber tube effected the seal between the rod and glass tube. This device disturbed the sucrose gradient only slightly when its tip was rapidly lowered to the vicinity of the agar surface and it permitted rapid transfer of the contents of the syringe to the electrophoresis cell. The resulting zone

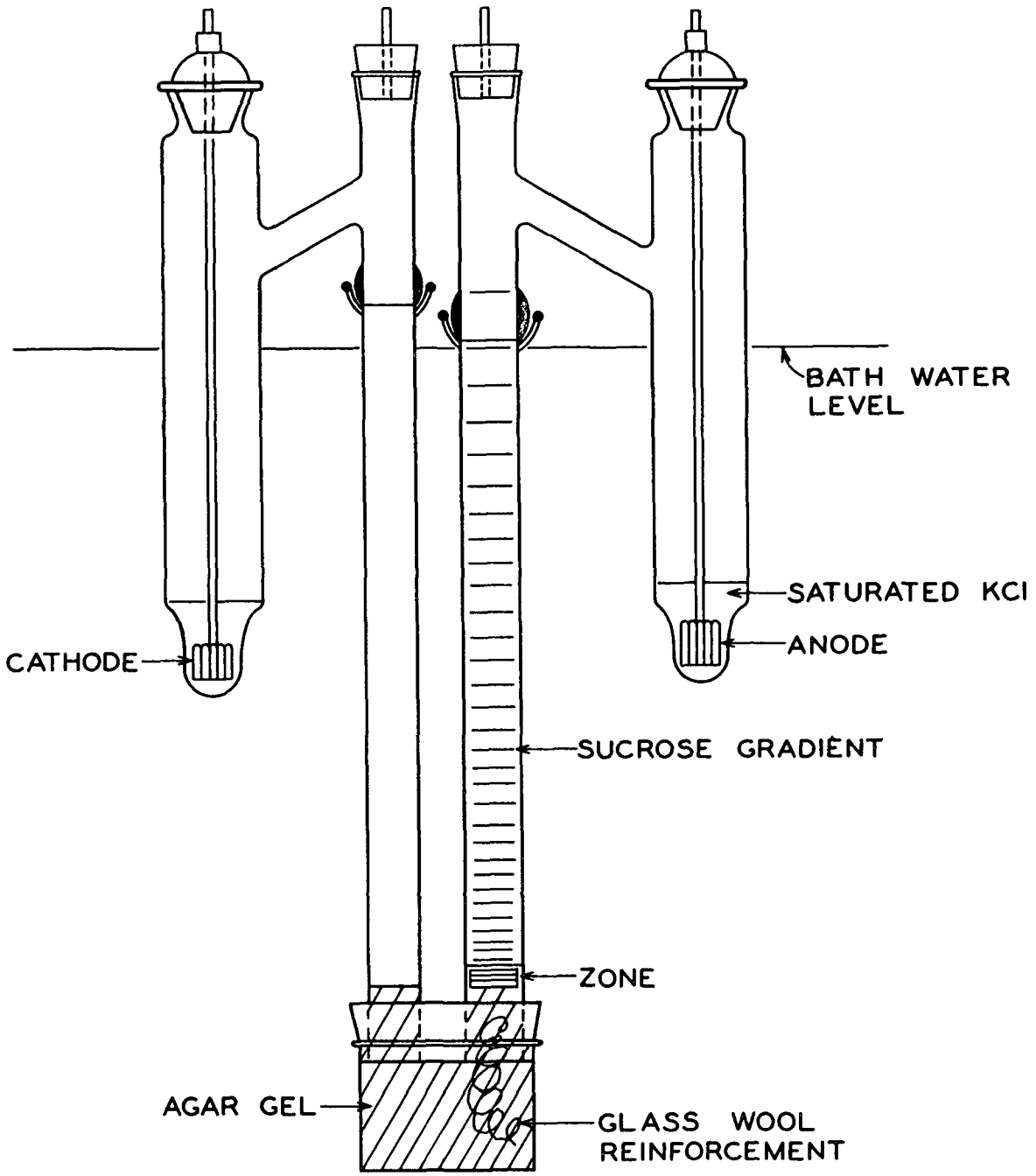


Figure 28. Zone Electrophoresis Cell Designed by Dillingham

was 8 to 12 mm. high. Electrophoresis for 30 hours at 30 ma. was followed by the collection of 14 to 18 fractions. The fractions were analyzed spectrophotometrically as follows: Ten λ of each fraction were added to 10 ml. of 0.25% catechol in buffer solution No. 4 at zero time. Fifteen minutes later, the percentage of transmission was read at 420 mμ on a Universal Spectrophotometer Model 14 manufactured by Coleman Instruments, Inc., Maywood, Illinois. Typical results were illustrated in Fig. 6 for preparations containing pseudolaccase and tyrosinase. Fractions were combined to yield one solution rich in pseudolaccase, one rich in tyrosinase, and one containing both enzymatic activities. These solutions were desalted, quick-frozen, and lyophilized prior to further work.

The dry preparation containing both enzymatic activities was combined with others like it and rerun. The solids rich in tyrosinase were set aside. The preparation rich in pseudolaccase was combined with others like it from additional runs and rerun twice, each time discarding the tail of materials which migrate slowly. A typical curve of percentage of transmission vs. fraction number for the final preparation K was shown in Fig. 7, page 29.

APPENDIX V

ANALYTICAL ELECTROPHORESIS PROCEDURES

Pseudolaccase K solutions of 0.4 to 0.6% concentration were used in all experiments to reduce optical errors inherent in measuring high refractive index gradients and to reduce the difference in buffer salt concentration between the enzyme solution and the buffer solvent. (The latter determines the size of the ghost boundary.) The conductivity of the buffer solution measured at the temperature of the electrophoresis instrument bath (ca. 1°C.) was used to calculate the field strength. The pH of buffer solutions was measured with a glass electrode.

Special care to avoid thermal convection must be taken in electrophoresis spreading experiments. Power loads which do not cause convection in short experiments (2 to 3 hours) may cause convection in prolonged spreading experiments (8 to 10 hours) as the density gradient becomes progressively less; therefore, the load was kept at 0.015 watt/ml. or less (10% of the routine load).

The experimental error arising from the breadth of the initial boundary as it effected the calculation of the apparent diffusion coefficient by the method of differences was not prohibitive, since the electrophoresis spreading was large for the system under investigation. Boundary sharpening by the technique of Kahn and Polson (87) should be used when spreading is small.

The optical system used in this research was the cylindrical lens schlieren optical system as supplied with the Model H Electrophoresis-

Diffusion Instrument without modification. The 2-ml. microcell was used. Photographs were taken on Eastman Kodak contrast process panchromatic film and developed for 4 minutes with Kodak D-11 at 76°F. The concentration gradient curves were measured on a Wilder Microprojector.

Electrophoresis spreading experiments at the ideal pH of the isoelectric point are often impossible due to loss of activity or insolubility of an enzyme. Well-buffered solutions at an ionic strength of approximately 0.1 suffice to sufficiently damp out charge effects resulting at pHs other than the isoelectric point of the enzyme under consideration. Prior to filling the Tiselius-type electrophoresis cell, the enzyme solution was centrifuged for 10 minutes at 10,000 times gravity to remove dust and aggregates of protein. The cell was assembled and filled according to procedures which have been carefully described in the literature (52).

APPENDIX VI

DETERMINATION OF HETEROGENEITY AND DIFFUSION COEFFICIENTS-- THEORY AND EXPERIMENTAL DETAILS

Experiments performed at the isoelectric point of an enzyme (providing it was stable and soluble) and at low field strength minimized all phenomena except diffusion and electrophoretic mobility. Other effects, such as pH and concentration gradients, etc., were negligible as shown by the near enantiography of the ascending and descending schlieren patterns.[a] Sharp, et al. (61) gave Equation (6) for the concentration gradient curve when the boundary spreading was due only to diffusion and a distribution of mobilities. The refractive index gradient, dn/dx, was taken as the height of an ordinate in the schlieren pattern in

$$\frac{dn}{dx} = \frac{n_1 - n_2}{2\sqrt{\pi Dt}} \int_{-\infty}^{+\infty} g(u)\, e^{-(x-uEt)^2/4Dt}\, du \qquad (6)$$

arbitrary units, x was the abscissa of the pattern, and $n_1 - n_2$, the refractive index change across the boundary, was taken as the area of the pattern, $\Delta x \Sigma dn/dx$, in corresponding units. E was the field strength, t was the time, D was the diffusion coefficient, and $g(u)$ was the mobility distribution function.

---

[a] The ascending schlieren pattern was the photographic record of the concentration gradient in the leg of the Tiselius-type electrophoresis cell where the mobile molecules were traveling vertically upward into solvent. The descending schlieren pattern was for those molecules traveling vertically downward into solution in the other leg of the Tiselius-type cell.

If diffusion was not negligible during the electrophoresis experiment, the usual case, it was theoretically possible but mathematically impractical to use Equation (6) to obtain the mobility distribution, $g(u)$ (56). A Gaussian distribution function has been closely realized by many systems involving natural proteins (57,58) at their respective "average" isoelectric points where the most frequent molecule had a mobility of zero. If a protein has a Gaussian distribution of mobilities and it was assumed that the electrophoretic species migrate and diffuse independently, the second moment, σ^2 , of the concentration gradient curve at time t was given by Equation (7). Second moments of

$$\sigma^2 = \sigma_o^2 + 2Dt + \beta^2E^2t^2 \qquad (7)$$

independent distributions add to give the second moment of the combined distribution. σ_o^2 was the second moment at the time the field was applied, $2Dt$ was the second moment for diffusion alone, and $\beta^2E^2t^2$ was the second moment for spreading due to electrophoretic heterogeneity. β was the standard deviation of the mobility distribution and was more commonly called the heterogeneity coefficient.

When Equation (7) was rewritten to give Equation (8), it was seen that β could be evaluated without knowing D, because $(\sigma^2-\sigma_o^2)/2t$ was a

$$D^* = \frac{\sigma^2 - \sigma_o^2}{2t} = D + \frac{\beta^2E^2}{2}t \qquad (8)$$

linear function of t, and β could be found from the slope of a plot of D^* versus t. D, the diffusion coefficient, was the intercept obtained by extrapolating to zero time. D^* was the apparent diffusion coefficient

calculated from the experimental concentration gradient curves obtained during electrophoresis. The apparent diffusion coefficient could be calculated from the concentration gradient curves by any of the standard methods, providing a Gaussian distribution of mobilities existed. The first half of Equation (8), which utilizes the second moment of the concentration gradient curve was used in this research. When second moments are used, β may be determined for proteins possessing both Gaussian and non-Gaussian distributions of mobilities (60). The simplest method of evaluating the second moment involved the relationship between A, the area under the concentration gradient curve, and $y_{max.}$, the maximum ordinate of the same curve. See Equation (9).

$$\sigma^2 = \frac{1}{2\pi}\left(\frac{A}{y_{max.}}\right)^2 \qquad (9)$$

The Spinco Model E Ultracentrifuge was also used to evaluate the diffusion coefficient of pseudolaccase. A boundary was initially formed near the center of a capillary-type synthetic boundary cell and the spreading of the boundary was studied at low speed. In this procedure, the boundary moved very slowly and spreading due to heterogeneity of molecular weight was reduced. Apparent diffusion coefficients were evaluated by using Equation (10) (52) and were plotted agains $1/t$ to correct for imperfections in the initial boundary. The correction

$$D^* = \frac{1}{4\pi t}\left(\frac{A}{y_{max.}}\right)^2 (1-\omega^2 st) \qquad (10)$$

factor, $(1-\omega^2 st)$ adjusted for the inhomogeneity of the centrifugal field as the distance between the boundary and its axis of rotation, slowly

increased. The true diffusion coefficient was found by an extrapolation to infinite time; i.e., $1/t$ equaled zero.

The second moments of the concentration gradient curves obtained from an ultracentrifuge run were plotted against time as an alternative procedure for obtaining the diffusion coefficient. The second moments were evaluated as described above. The slope of the straight line obtained was equal to $2D$. The accuracy of diffusion coefficients determined in this manner was comparable with those determined in the Tiselius-type electrophoresis cell when schlieren optics were employed.

APPENDIX VII

MICHAELIS-MENTEN THEORY AND INTERPRETATION

The Michaelis-Menten theory (65) was based on the assumption that the enzyme forms a reversibly dissociable complex with the substrate. Instantaneous equilibrium of such complex formation is presumed. Equations (5) and (11) are algebraic forms of the above statements.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (5)$$

and

$$\frac{d(ES)}{dt} = 0 \qquad (11)$$

The rate of formation of ES may be written,

$$\frac{d(ES)}{dt} = k_1[(E_t)-(ES)](S) - (k_2 + k_3)(ES) \qquad (12)$$

where

$$(E_t) = (E) + (ES) \qquad (13)$$

The rate of disappearance of substrate may be written,

$$-\frac{d(S)}{dt} = k_1[(E_t) - (ES)](S) - k_2(ES) \qquad (14)$$

The over-all rate of formation of product is equal to the difference between Equations (14) and (12):

$$\frac{d(P)}{dt} = -\frac{d(ES)}{dt} - \frac{d(S)}{dt} = k_3(ES) \qquad (15)$$

which, in light of Equation (11), reduces to

$$\frac{d(P)}{dt} = -\frac{d(S)}{dt} = k_3(ES) \qquad (16)$$

Since $\frac{d(ES)}{dt} = 0$,

$$k_1[(E_t) - (ES)](S) = (k_2 + k_3)(ES) \qquad (17)$$

and

$$K_m = \frac{k_2 + k_3}{k_1} = \frac{[(E_t) - (ES)](S)}{(ES)} = \frac{(E)(S)}{(ES)} \qquad (18)$$

Solving Equation (18) for (ES) and substituting into Equation (16) gives Equation (19):

$$v = -\frac{d(S)}{dt} = \frac{k_3(E_t)(S)}{K_m + (S)} \qquad (19)$$

According to the first-order mass law, ($\underline{ES}$) increases as ($\underline{S}$) is increased and $\underline{v}$ increases hyperbolically to $\underline{V}$, the maximum velocity of a reaction at any set of given conditions. Therefore, Equation (19) becomes

$$v = \frac{V(S)}{K_m + (S)} \qquad (20)$$

where $V = k_3(E_t)$. (21)

Equation (20) was rearranged by Lineweaver and Burk (64) to give a straight-line plot of $1/\underline{v}$ versus $1/(\underline{S})$

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V(S)} \qquad (22)$$

It is evident that interpretation of the kinetic data of enzymatic reactions, by first-order kinetics will lead to erroneous results unless

the substrate concentration is decreased sufficiently to render the rate of combination of enzyme and substrate the rate-limiting step. At higher substrate concentrations only the integrated form of Equation (19) will apply:

$$k_3(E_t)t = K_m \ln \frac{S_o}{S} + (S_o - S). \qquad (23)$$

The reaction rate kinetics for the Michaelis-Menten theory will be first order when $\underline{K_m}$ is sufficiently great to cause the equilibrium concentration of $(\underline{ES})$ to be small compared to $(\underline{E_t})$, or when $(\underline{S})$ is sufficiently small to render the first-order term of Equation (23) predominant in comparison to the zero order term.

APPENDIX VIII

BERNSTEIN-BALLENTINE PROPORTIONAL COUNTING TUBE CALIBRATION

Three Bernstein-Ballentine (68) proportional gas phase counting tubes supplied by Nuclear Instrument and Chemical Corporation of Chicago, Illinois were characterized. The background count and counting plateaus were determined as a function of the applied voltage. Figure 29 depicts the trend of the data which was normal. The background count was approximately 165 counts per minute for each tube at 3.8 kv.

The counting rate at constant C^{14} content as a function of the total carbon content over a range of voltages was also characterized. Figure 30 graphically summarizes the data presented in Table XIV. The usual loss of counting efficiency in proportion to the carbon content at carbon contents greater than 7 mg. was not found. Ten λ of a coniferyl alcohol solution (0.2018 mg./ml.) were added to circles of Whatman No. 1 chromatography paper containing approximately 4.6, 9.0, 13.0, and 17.5 mg. of carbon. The circles were subjected to wet combustion, the carbon content checked manometrically, and the carbon dioxide gas transferred to the evacuated counting tube with its tip in liquid nitrogen. At 3.8 kv., all three tubes gave a specific activity of 904 counts per minute per γ of coniferyl alcohol plus or minus 17 counts per minute per γ. Total counts were 10,000 or greater, therefore giving a standard error of counting of 1% or less.

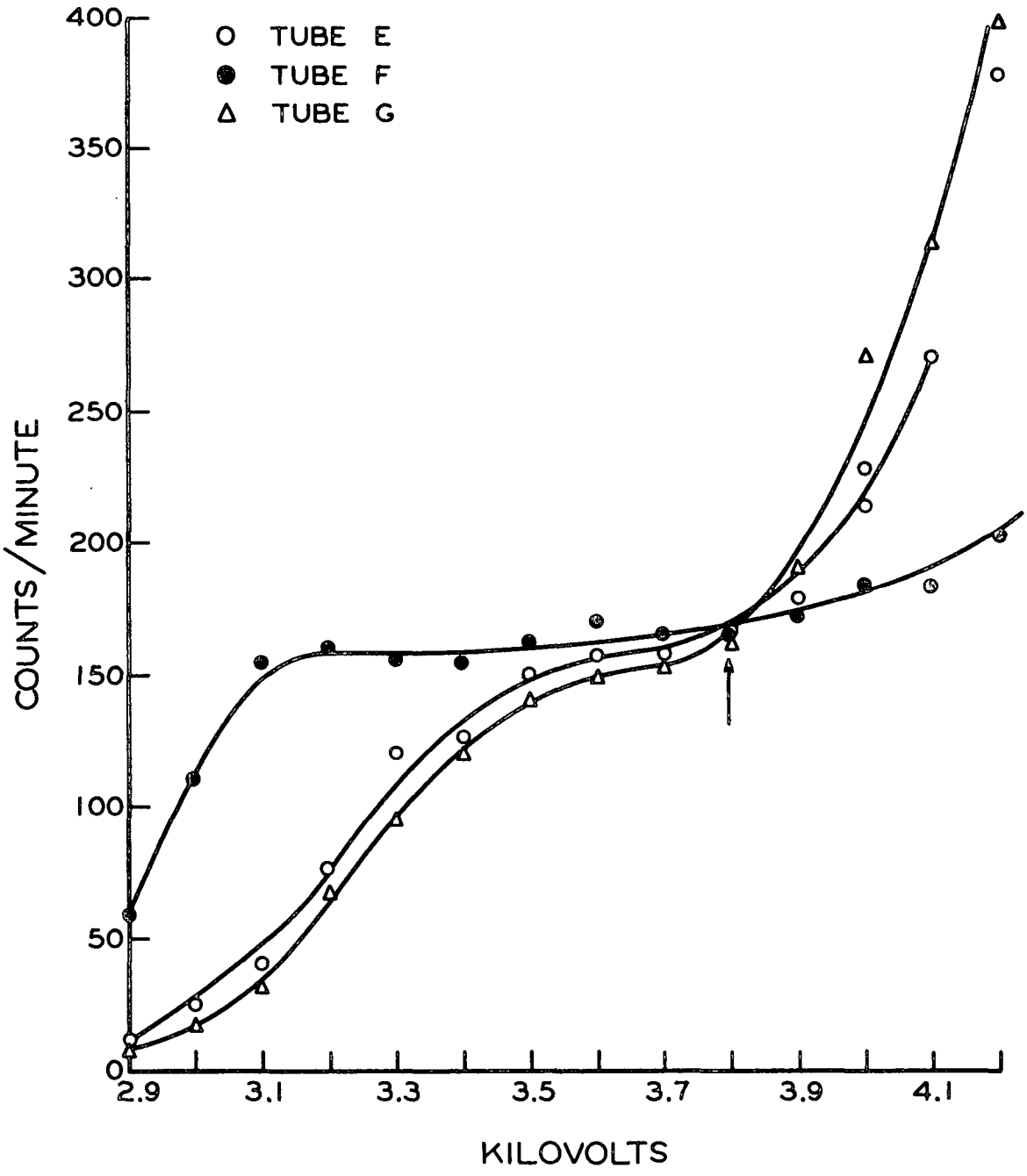


Figure 29. Background Count for Bernstein-Ballentine Proportional Counting Tubes as a Function of Voltage

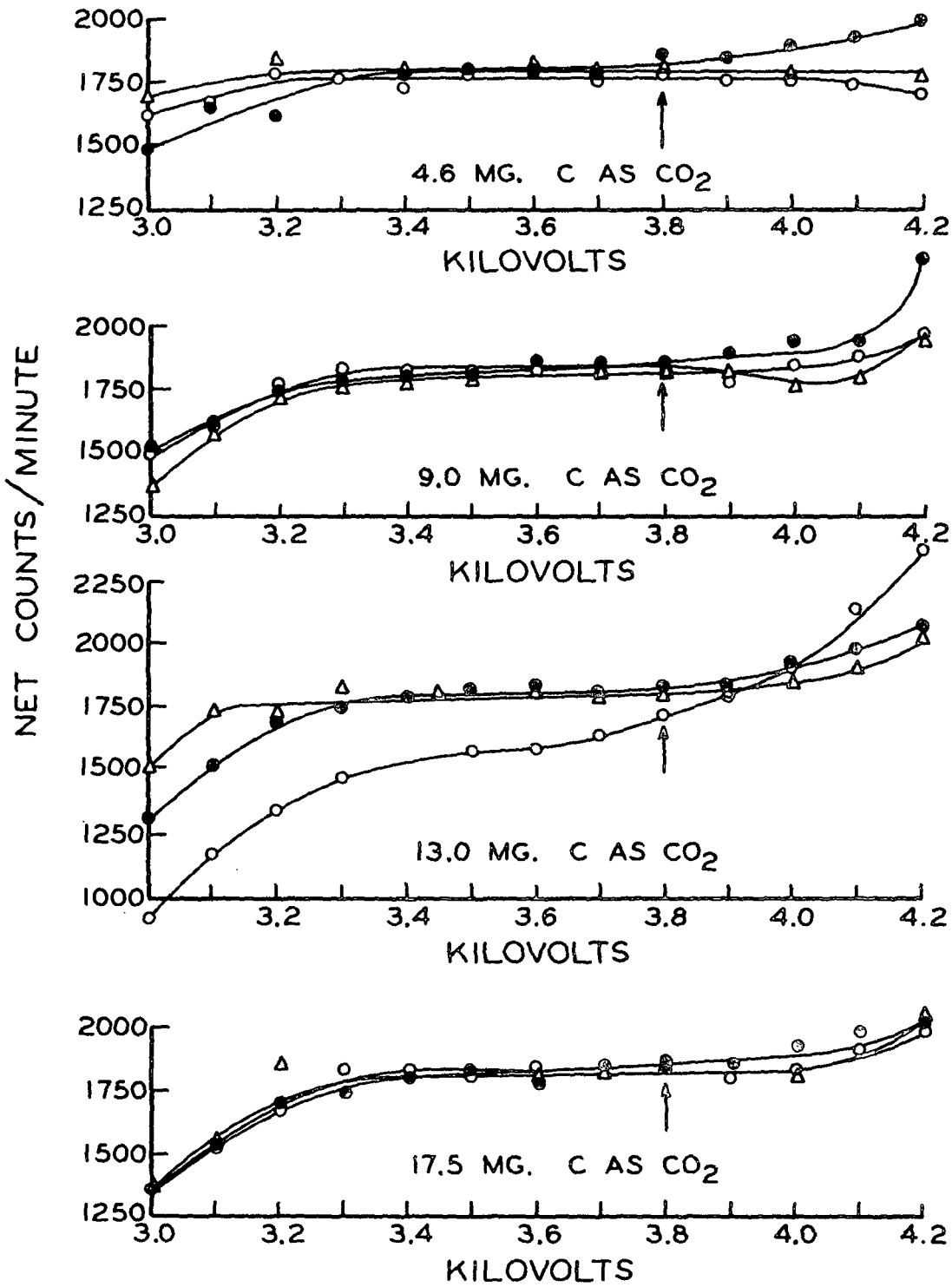


Figure 30. Counting Rates for Bernstein-Ballentine Proportional Counting Tubes as a Function of Voltage and Carbon Dioxide Content
o Tube E; ● Tube F; Δ Tube G

TABLE XIV

COUNTING RATE (c.p.m.) AS A FUNCTION OF TUBE, VOLTAGE, AND TOTAL CARBON CONTENT

(Level of radioactivity constant at ca. 1824 c.p.m.)

Applied Voltage, kv.	Total Carbon 4.6 mg. Tube			Total Carbon 9.0 mg. Tube			Total Carbon 13.0 mg. Tube			Total Carbon 17.5 mg. Tube		
	E	F	G	E	F	G	E	F	G	E	F	G
3.0	1627	1480	1694	1502	1514	1380	928	1320	1504	1335	1372	1364
3.1	1680	1656	--	1610	1617	1578	1175	1528	1728	1518	1525	1570
3.2	1789	1616	1842	1772	1735	1718	1353	1682	1734	1673	1693	1867
3.3	1765	1768	--	1827	1777	1756	1472	1744	1826	1825	1749	--
3.4	1729	1784	1805	1813	1802	1782	--	1788	1813	1813	1784	1767
3.5	1778	1807	--	1799	1810	1800	1576	1817	--	1788	1821	--
3.6	1795	1798	1822	1821	1848	1841	1585	1833	1812	1834	1786	1810
3.7	1759	1781	1792	1820	1842	1824	1640	1806	1800	1809	1856	1836
3.8	1775	1863	1806	1826	1854	1816	1721	1877	1803	1839	1851	1865
3.9	1757	1848	--	1775	1885	1818	1793	1842	1818	1798	1870	--
4.0	1748	1882	1786	1850	1929	1764	1898	1929	1853	1835	1943	1828
4.1	1754	1935	--	1876	1935	1794	2143	1981	1904	1900	1994	--
4.2	1700	1991	1773	1960	2264	1958	2368	2059	2045	1978	--	2065

APPENDIX IX

INITIAL REACTION VELOCITY DATA

Data for the determination of initial reaction velocities, v, from oxygen absorption and radiotracer experiments are presented in graphical and tabular form.

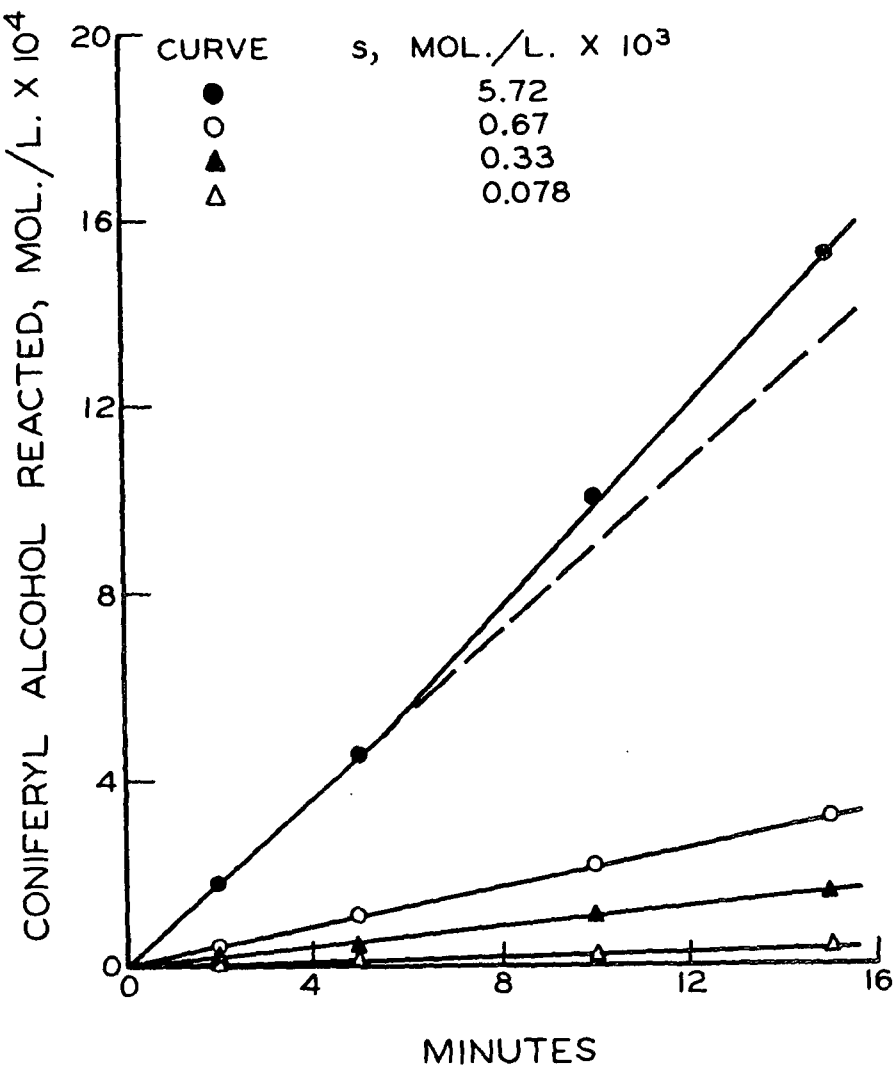


Figure 31. Moles of Coniferyl Alcohol-C^{14} Reacted Versus Time for the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.2

TABLE XV

REACTION RATE DATA FOR THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.2, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^{3}	1/s, liters/mole x 10^{-3}	Time, minutes	Coniferyl Alcohol, moles/liter x 10^{4}	Initial Rates Coniferyl Alcohol, moles/liter-minute x 10^{5}	1/v, coniferyl alcohol liter-minutes/mole x 10^{-4}
5.72 (1)[a]	0.175	2	1.81	9.1	1.09
		5	4.58		
		10	10.01		
		15	15.24		
0.67 (2)	1.490	2	0.42	2.08	4.81
		5	1.07		
		10	2.16		
		15	3.21		
0.33 (3)	3.030	2	0.22	1.03	9.70
		5	0.54		
		10	1.09		
		15	1.64		
0.078 (4)	12.82	2	0.05	0.22	45.50
		5	0.14		
		10	0.27		
		15	0.41		

[a]Number in parentheses refers to the respective curve in Fig. 31.

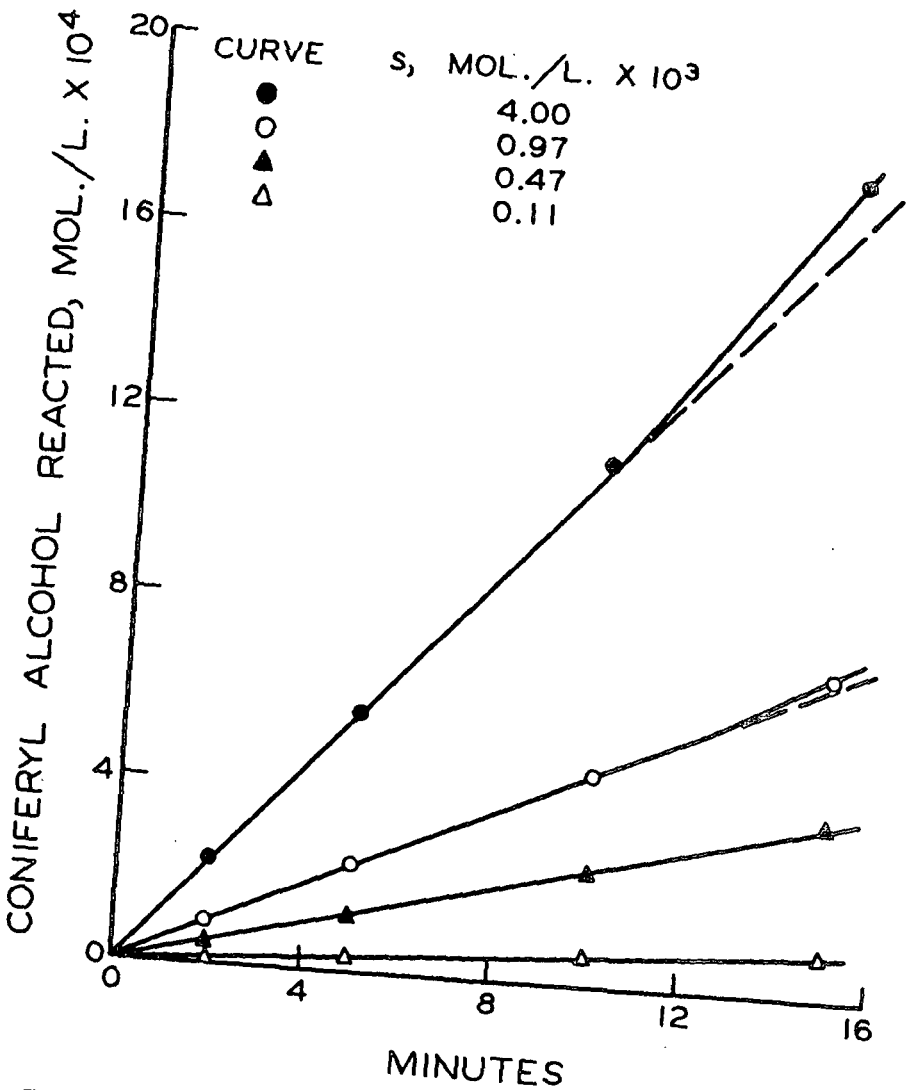


Figure 32. Moles of Coniferyl Alcohol-C^{14} Reacted Versus Time for the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.7

TABLE XVI

REACTION RATE DATA FOR THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.7, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^3	1/s, liters/mole x 10^{-3}	Time, minutes	Coniferyl Alcohol, moles/liter x 10^4	Initial Rates: Coniferyl Alcohol, moles/liter-minute x 10^5	1/v, coniferyl alcohol liter-minutes/mole x 10^{-4}
4.00 (1)[a]	0.250	2	2.28	11.1	0.90
		5	5.62		
		10	11.26		
		15	17.60		
0.97 (2)	1.033	2	0.91	4.5	2.22
		5	2.28		
		10	4.56		
		15	6.94		
0.47 (3)	2.118	2	0.50	2.48	4.03
		5	1.24		
		10	2.49		
		15	3.74		
0.11 (4)	8.78	2	0.13	0.61	16.40
		5	0.32		
		10	0.64		
		15	0.96		

[a] Number in parentheses refers to the respective curve in Fig. 32.

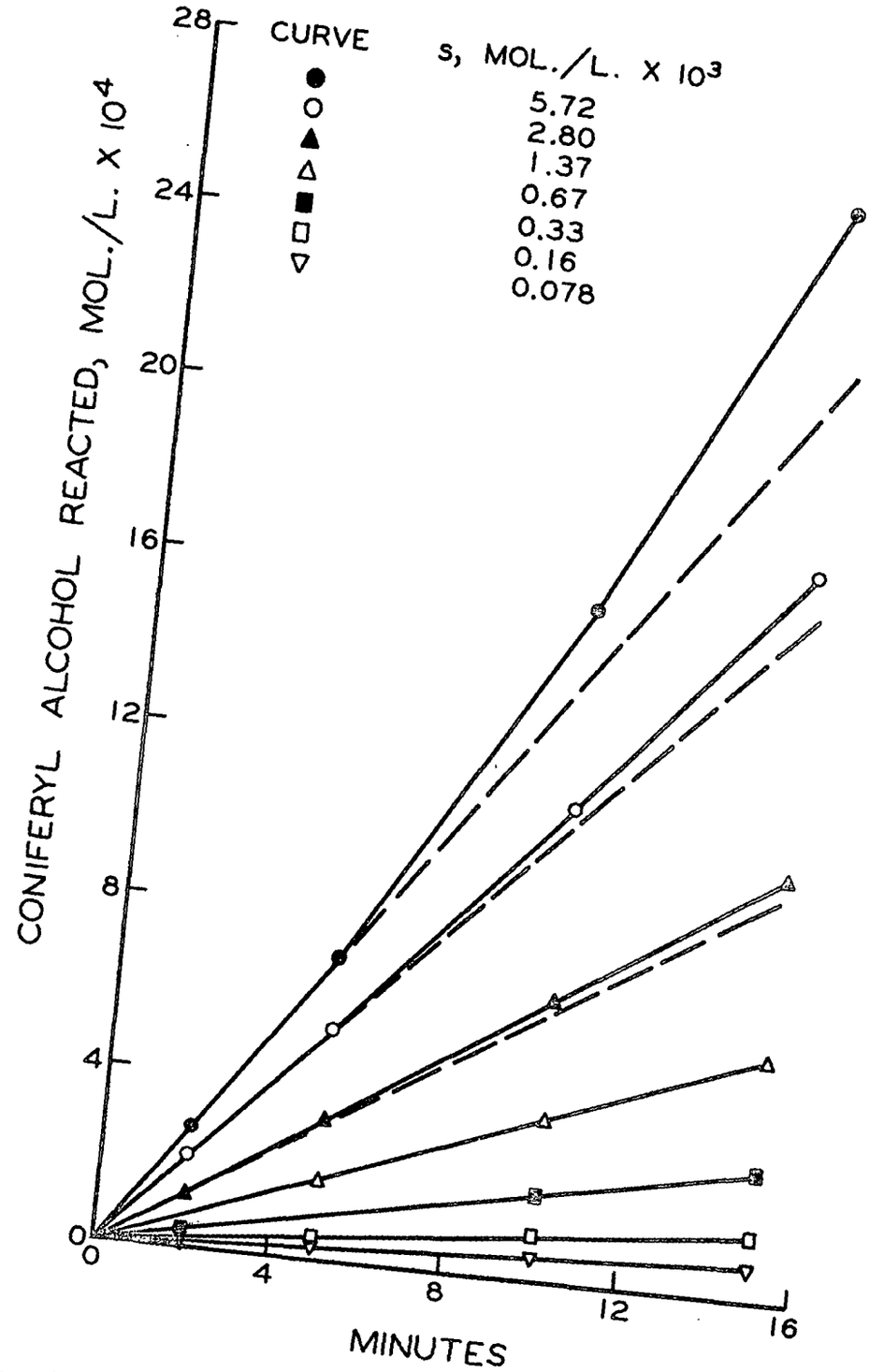


Figure 33. Moles of Coniferyl Alcohol-C^{14} Reacted Versus Time for the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.2

TABLE XVII

REACTION RATE DATA FOR THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.2, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^3	1/s, liters/mole x 10^{-3}	Time, minutes	Coniferyl Alcohol, moles/liter x 10^4	Initial Rates Coniferyl Alcohol, moles/liter-minute x 10^5	1/v, coniferyl alcohol liter-minutes/mole x 10^{-4}
5.72 (1)[a]	0.175	2	2.76	13.9	0.72
		5	6.94		
		10	15.50		
		15	25.18		
2.80 (2)	0.357	2	2.10	10.5	0.95
		5	5.13		
		10	10.87		
		15	16.72		
1.37 (3)	0.730	2	1.27	6.2	1.61
		5	3.22		
		10	6.47		
		15	9.71		
0.67 (4)	1.490	2	--	3.68	2.72
		5	1.83		
		10	3.69		
		15	5.55		
0.33 (5)	3.030	2	0.39	1.92	5.21
		5	--		
		10	1.95		
		15	2.93		
0.16 (6)	6.250	2	0.20	1.00	10.00
		5	0.50		
		10	1.00		
		15	1.50		
0.078 (7)	12.820	2	0.105	0.50	20.00
		5	0.256		
		10	0.516		
		15	0.780		

[a]Number in parentheses refers to the respective curve in Fig. 33.

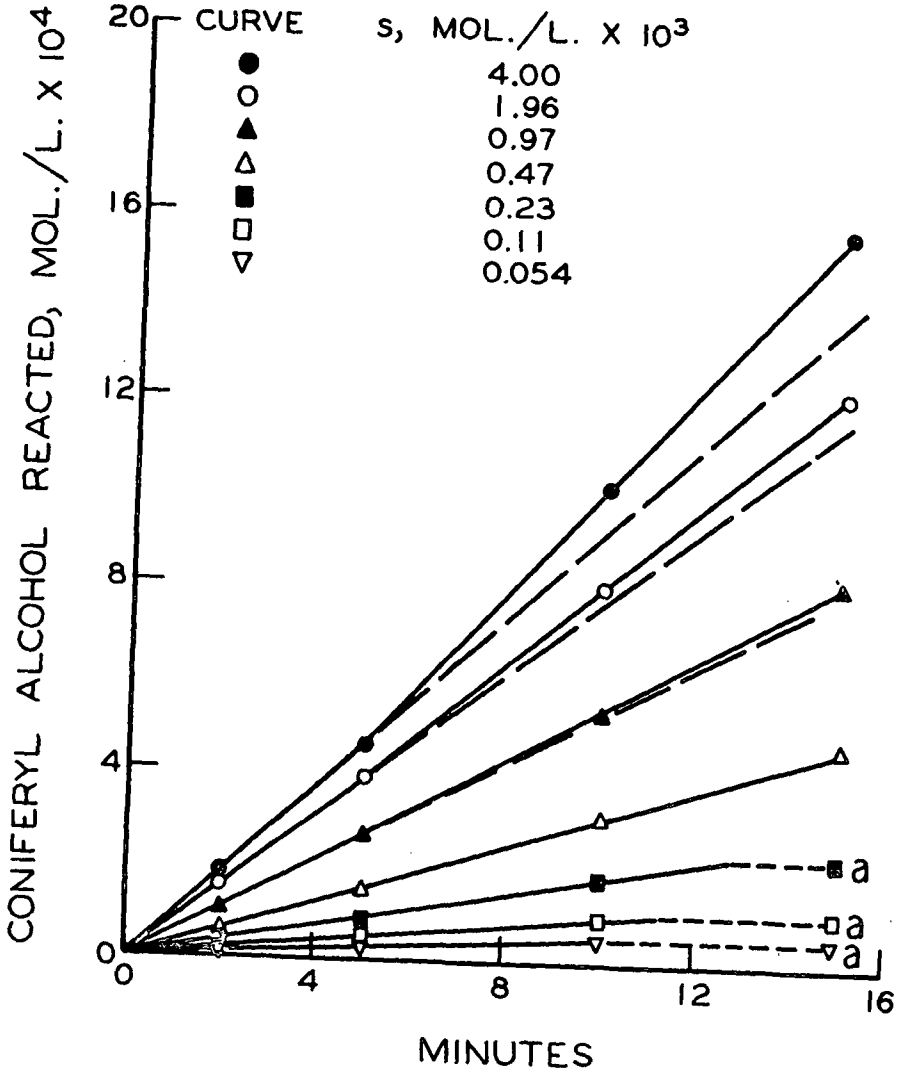


Figure 34. Moles of Coniferyl Alcohol-C^{14} Reacted Versus Time for the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.7

[a]The horizontal broken line connects the point on the solid curve where 100% of the coniferyl alcohol had reacted with the value measured at 15 minutes.

TABLE XVIII

REACTION RATE DATA FOR THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.7, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^3	1/s, liters/mole x 10^{-3}	Time, minutes	Coniferyl Alcohol, moles/liter x 10^4	Initial Rates: Coniferyl Alcohol, moles/liter-minute x 10^5	1/v, coniferyl alcohol liter-minutes/mole x 10^{-4}
4.00 (1)[a]	0.250	2	1.84	9.22	1.09
		5	4.60		
		10	10.15		
		15	15.68		
1.96 (2)	0.510	2	1.54	7.75	1.29
		5	3.87		
		10	8.06		
		15	12.23		
0.97 (3)	1.033	2	1.07	5.30	1.89
		5	2.67		
		10	5.35		
		15	8.11		
0.47 (4)	2.118	2	0.62	3.10	3.22
		5	1.58		
		10	3.16		
		15	4.70		
0.23 (5)	4.305	2	0.36	1.81	5.52
		5	0.90		
		10	1.80		
		15	2.30		
0.11 (6)	8.780	2	0.20	0.97	10.31
		5	0.49		
		10	0.98		
		15	1.10		
0.054 (7)	18.35	2	0.11	0.53	18.88
		5	0.26		
		10	0.53		
		15	0.54		

[a]Number in parentheses refers to the respective curve in Fig. 34.

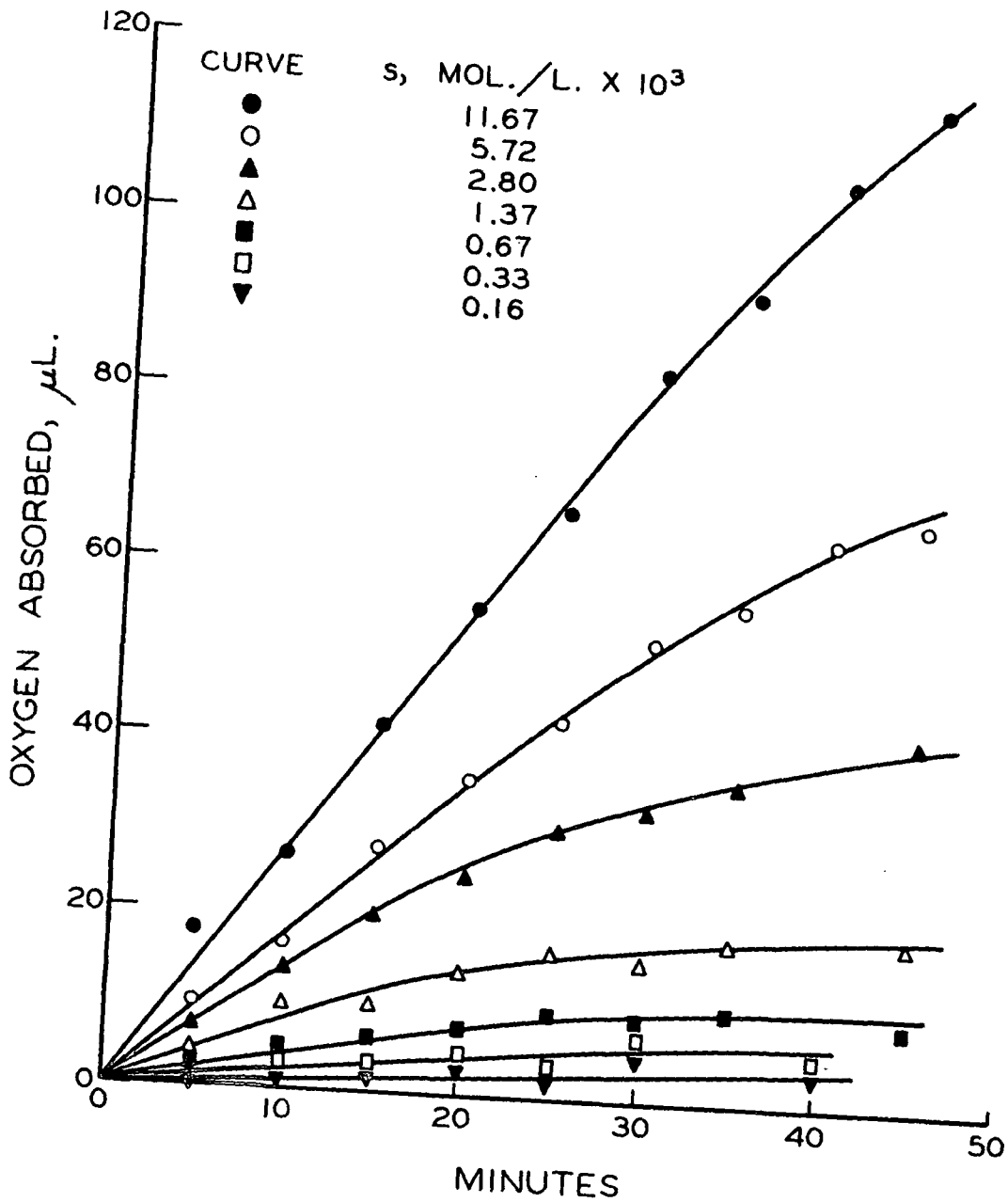


Figure 35. Oxygen Absorption-Time Curves for the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.2, at 25°C.

TABLE XIX

REACTION RATE DATA FOR THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.2, AT 25°C. AS OBTAINED BY OXYGEN ABSORPTION MEASUREMENTS USING A WARBURG CONSTANT VOLUME RESPIROMETER

Substrate Concentration, s, moles/liter x 10^3	1/s, liters/mole x 10^{-3}	Time, minutes	Oxygen Absorbed, µl.[a]	Initial Rates: Oxygen Used, µl./3 ml./minute	Initial Rates: Coniferyl Alcohol, moles/liter-minute x 10^5	1/v, coniferyl alcohol liter-minutes/mole x 10^{-4}
11.67 (1)[b]	0.086	5	17.6	2.76	15.00	0.67
		10	26.5			
		15	41.6			
		20	55.5			
		25	66.6			
		35	92.6			
		45	114.7			
5.72 (2)	0.175	5	9.4	1.79	9.80	1.02
		10	16.7			
		15	26.9			
		20	36.0			
		25	42.9			
		35	56.1			
		45	66.3			
2.80 (3)	0.357	5	6.5	1.39	7.52	1.33
		10	13.6			
		15	20.0			
		20	24.7			
		25	30.3			
		35	36.2			
		45	41.8			
1.37 (4)	0.730	5	3.9	0.76	4.08	2.45
		10	9.4			
		15	9.5			
		20	13.7			
		25	16.1			
		35	17.9			
		45	18.6			
0.67 (5)	1.490	5	2.0	0.37	1.96	5.10
		10	4.7			
		15	5.9			
		20	7.3			
		25	9.5			
		35	10.0			
		45	9.1			
0.33 (6)	3.030	5	1.3	0.20	1.14	8.80
		10	2.9			
		15	3.1			
		20	4.7			
		25	3.4			
		30	7.0			
		40	5.4			
0.16 (7)	6.250	5	0.4	0.10	0.54	18.50
		10	1.1			
		15	1.3			
		20	2.6			
		30	4.3			
		40	2.9			

[a] Average of three determinations.
[b] Number in parentheses refers to the respective curve in Fig. 35.

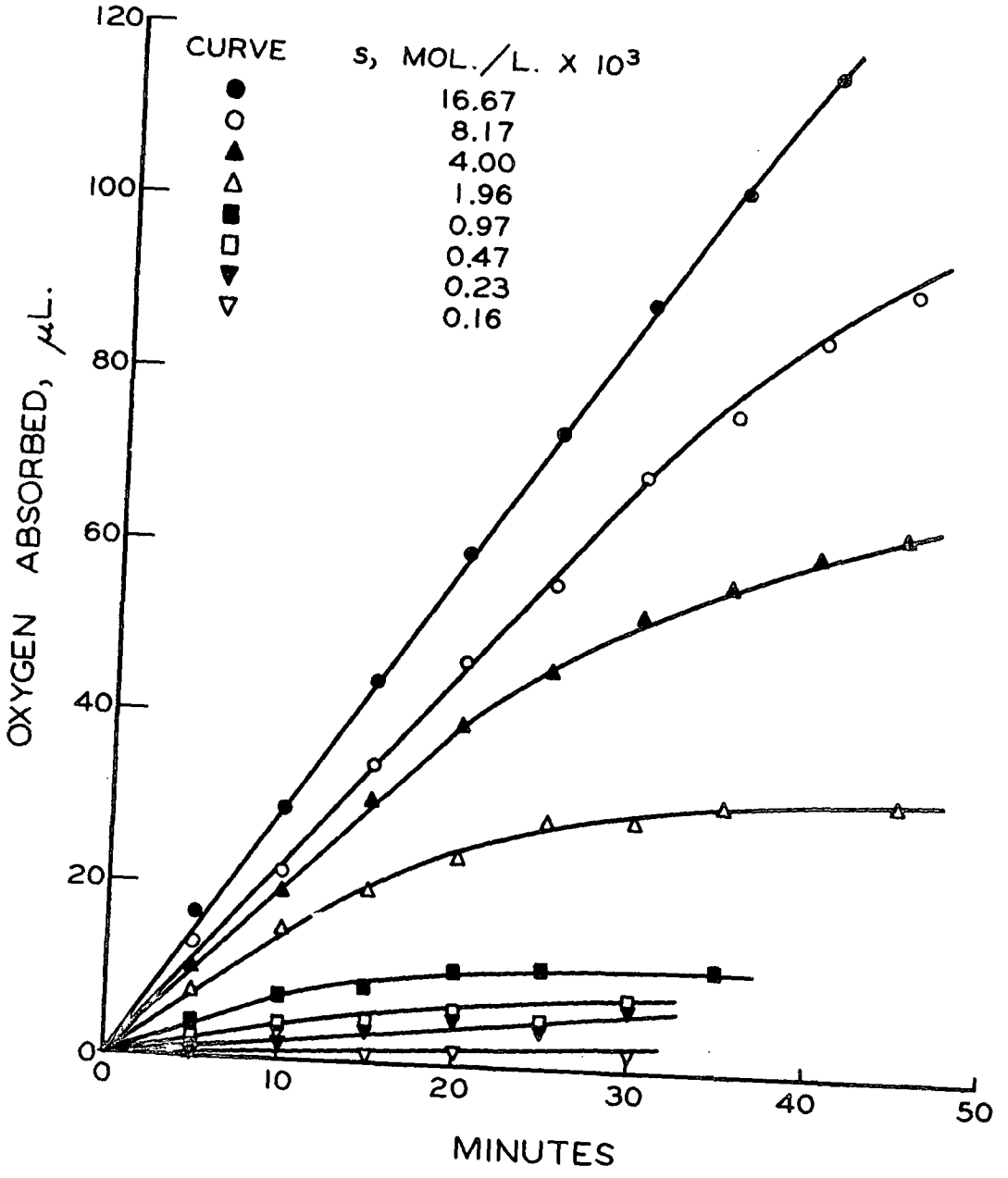


Figure 36. Oxygen Absorption-Time Curves for the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.7, at 25°C.

TABLE XX

REACTION RATE DATA FOR THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.7, AT 25°C. AS OBTAINED BY OXYGEN ABSORPTION MEASUREMENTS USING A WARBURG CONSTANT VOLUME RESPIROMETER

Substrate Concentration, s, moles/liter x 10^3	$1/s$, liters/mole x 10^{-3}	Time, minutes	Oxygen Absorbed, µl.[a]	Initial Rates: Oxygen Used, µl./3 ml./minute	Initial Rates: Coniferyl Alcohol, moles/liter-minute x 10^5	$1/v$, coniferyl alcohol liter-minutes/mole x 10^{-4}
16.67 (1)[b]	0.060	5	16.6	2.99	16.32	0.61
		10	29.1			
		15	44.1			
		20	59.5			
		30	89.3			
		40	116.8			
8.17 (2)	0.122	5	13.1	2.30	12.56	0.80
		10	22.0			
		15	34.6			
		20	47.0			
		25	56.0			
		35	76.6			
		45	91.1			
4.00 (3)	0.250	5	10.5	2.00	10.92	0.92
		10	19.4			
		15	30.2			
		20	39.0			
		25	46.2			
		35	56.6			
		45	62.3			
1.96 (4)	0.510	5	7.5	1.40	7.68	1.30
		10	15.0			
		15	19.6			
		20	24.0			
		30	28.7			
		45	31.6			
0.97 (5)	1.033	5	3.6	0.72	3.92	2.55
		10	7.2			
		15	8.6			
		20	10.8			
		25	11.3			
		35	12.1			
0.47 (6)	2.118	5	1.8	0.41	2.27	4.40
		10	4.0			
		15	4.5			
		20	6.2			
		25	5.3			
		30	8.0			
0.23 (7)	4.305	5	1.2	0.22	1.21	8.25
		10	1.9			
		15	3.8			
		20	5.1			
		25	4.3			
		30	6.1			
0.11 (8)	8.780	5	0.4	0.12	0.62	16.20
		15	0.6			
		20	1.2			
		30	1.8			

[a] Average of three determinations.

[b] Number in parentheses refers to the respective curve in Fig. 36.

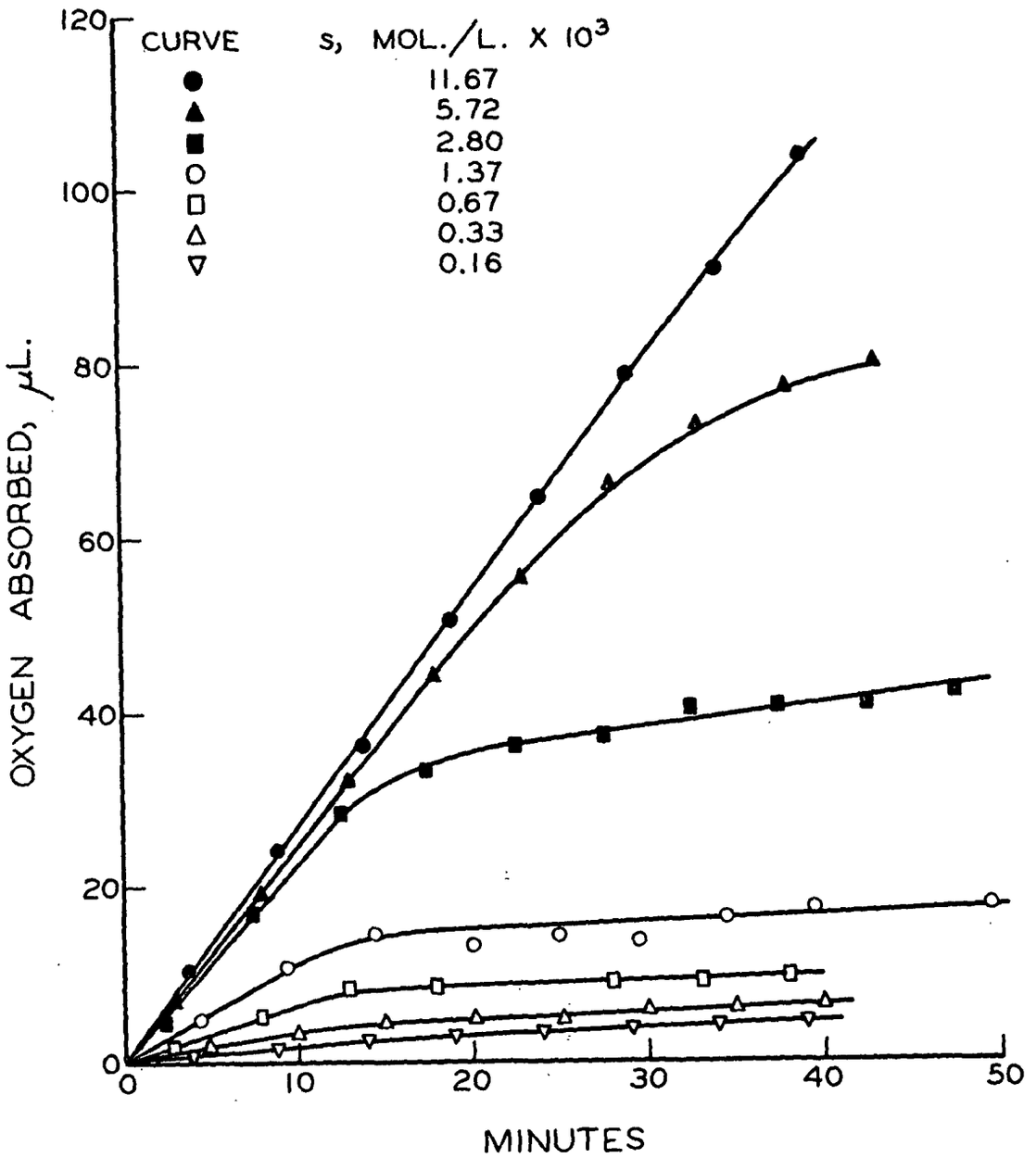


Figure 37. Oxygen Absorption-Time Curves for the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.2, at 25°C.

TABLE XXI

REACTION RATE DATA FOR THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.2, AT 25°C. AS OBTAINED BY OXYGEN ABSORPTION MEASUREMENTS USING A WARBURG CONSTANT VOLUME RESPIROMETER

Substrate Concentration, s, moles/liter x 10^3	1/s, liters/mole x 10^{-3}	Time, minutes	Oxygen Absorbed, µl.[a]	Initial Rates: Oxygen Used, µl./3 ml./minute	Initial Rates: Coniferyl Alcohol, moles/liter-minute x 10^5	1/v, coniferyl alcohol liter-minutes/mole x 10^{-4}
11.67 (1)[b]	0.086	4	10.4	2.72	14.8	0.68
		9	24.0			
		14	36.5			
		19	50.8			
		24	65.0			
		29	79.3			
		39	104.1			
5.72 (2)	0.175	3	7.1	2.52	13.6	0.74
		8	19.3			
		13	32.3			
		18	33.2			
		23	55.8			
		33	73.6			
		43	80.2			
2.80 (3)	0.357	3	4.8	2.26	12.8	0.78
		8	17.0			
		13	28.8			
		18	33.6			
		23	36.1			
		28	37.7			
		38	40.9			
		48	42.2			
1.37 (4)	0.730	4	4.9	1.15	6.24	1.60
		9	10.7			
		14	14.4			
		19	13.0			
		24	14.2			
		29	13.8			
		39	17.4			
0.67 (5)	1.490	3	2.0	0.66	3.60	2.78
		8	5.1			
		13	8.3			
		18	8.7			
		28	9.1			
		38	9.5			
0.33 (6)	3.030	5	1.8	0.35	1.89	5.28
		10	3.5			
		15	4.3			
		20	4.8			
		25	4.8			
		30	5.4			
		40	5.6			
0.16 (7)	6.250	4	0.7	0.18	0.96	10.4
		9	1.7			
		14	2.1			
		19	2.4			
		29	3.4			
		39	4.2			

[a] Average of three determinations.

[b] Number in parentheses refers to the respective curve in Fig. 37.

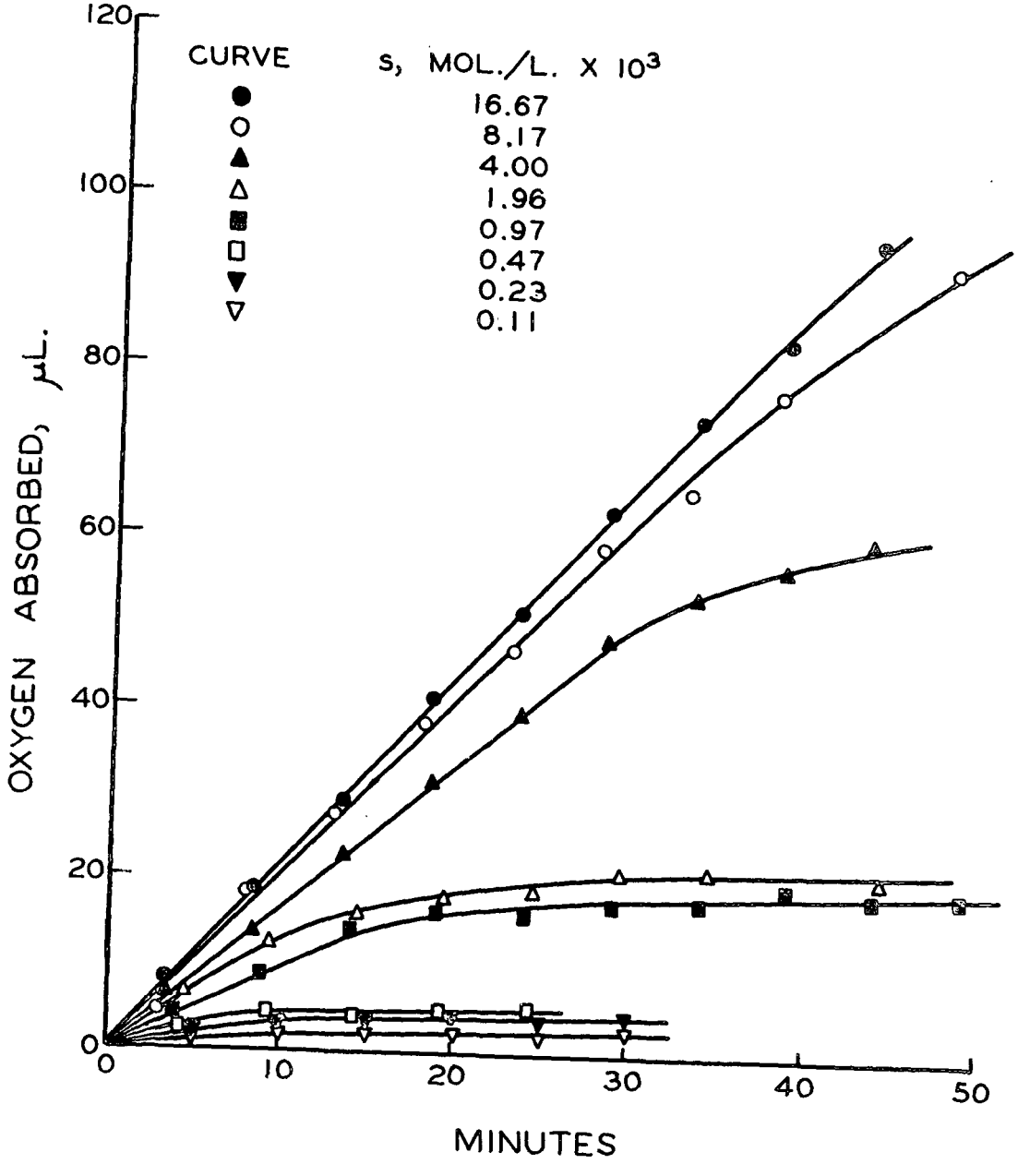


Figure 38. Oxygen Absorption-Time Curves for the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.7, at 25°C.

TABLE XXII

REACTION RATE DATA FOR THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.7, AT 25°C. AS OBTAINED BY OXYGEN ABSORPTION MEASUREMENTS USING A WARBURG CONSTANT VOLUME RESPIROMETER

Substrate Concentration, s, moles/liter x 10^3	$1/s$, liters/mole x 10^{-3}	Time, minutes	Oxygen Absorbed, µl.[a]	Initial Rates: Oxygen Used, µl./3 ml./minute	Initial Rates: Coniferyl Alcohol, moles/liter-minute x 10^5	$1/v$, coniferyl alcohol liter-minutes/moles x 10^{-4}
16.67 (1)[b]	0.060	3	8.2	2.21	12.04	0.83
		8	18.7			
		13	29.2			
		18	41.2			
		23	51.7			
		33	74.4			
		43	95.5			
8.17 (2)	0.122	3	4.0	2.10	11.44	0.87
		8	18.3			
		13	27.9			
		18	38.3			
		28	59.1			
		38	78.7			
4.00 (3)	0.250	3	6.7	1.70	9.28	1.08
		8	13.8			
		13	23.0			
		18	31.4			
		23	39.5			
		33	53.8			
		43	60.3			
1.96 (4)	0.510	4	6.8	1.40	7.64	1.31
		9	12.2			
		14	15.8			
		19	17.9			
		24	18.9			
		34	21.4			
0.97 (5)	1.033	4	4.1	0.97	5.28	1.90
		9	8.9			
		14	14.2			
		19	16.3			
		24	15.9			
		34	17.9			
		44	18.7			
0.47 (6)	2.118	4	2.4	0.58	3.16	3.16
		9	4.1			
		14	3.9			
		19	4.5			
		24	5.0			
0.23 (7)	4.305	5	1.7	0.33	1.80	5.56
		10	3.3			
		15	3.6			
		20	4.2			
		30	4.5			
0.11 (8)	8.780	5	0.9	0.18	0.96	10.4
		10	1.8			
		15	1.9			
		20	1.9			
		30	2.2			

[a] Average of three determinations.

[b] Number in parentheses refers to the respective curve in Fig. 38.

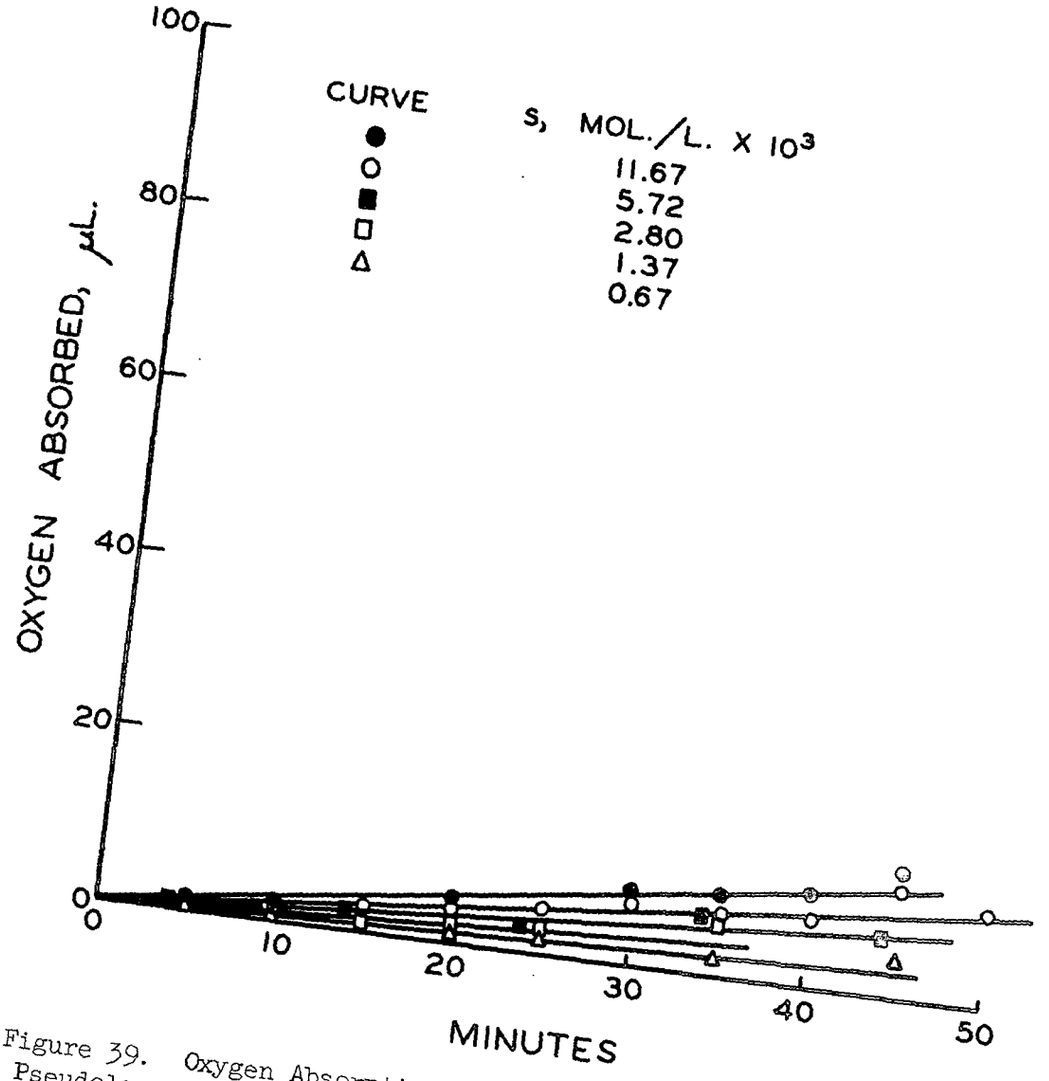


Figure 39. Oxygen Absorption-Time Curves for the p-Coumaryl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.2, at 25°C.

TABLE XXIII

REACTION RATE DATA FOR THE p-COUMARYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.2, AT 25°C. AS OBTAINED BY OXYGEN ABSORPTION MEASUREMENTS USING A WARBURG CONSTANT VOLUME RESPIROMETER

Substrate Concentration, s, moles/liter x 10^{3}	1/s, liters/mole x 10^{-3}	Time, minutes	Oxygen Absorbed, µl.[a]	Initial Rates: Oxygen Used, µl./3 ml./minute	Initial Rates: p-Coumaryl Alcohol, moles/liter-minute x 10^{5}	1/v, coniferyl alcohol liter-minutes/moles x 10^{-4}
11.67 (1)[b]	0.086	5	1.3	0.26	1.42	7.03
		10	3.1			
		20	5.1			
		30	8.5			
		35	9.2			
		40	10.6			
		45	14.3			
5.72 (2)	0.175	15	2.9	0.20	1.09	9.15
		20	3.8			
		25	5.3			
		30	6.9			
		35	7.0			
		40	7.6			
		50	10.3			
2.80 (3)	0.357	4	1.1	0.155	0.85	11.8
		9	1.4			
		14	2.1			
		24	2.8			
		34	6.9			
		44	6.4			
1.37 (4)	0.730	10	1.1	0.10	0.55	18.1
		15	0.7			
		20	2.1			
		25	2.7			
0.67 (5)	1.490	5	0.4	0.06	0.33	30.6
		10	0.5			
		20	1.1			
		25	1.6			
		35	2.7			
		45	5.0			

[a] Average of three determinations.

[b] Number in parentheses refers to the respective curve in Fig. 39.

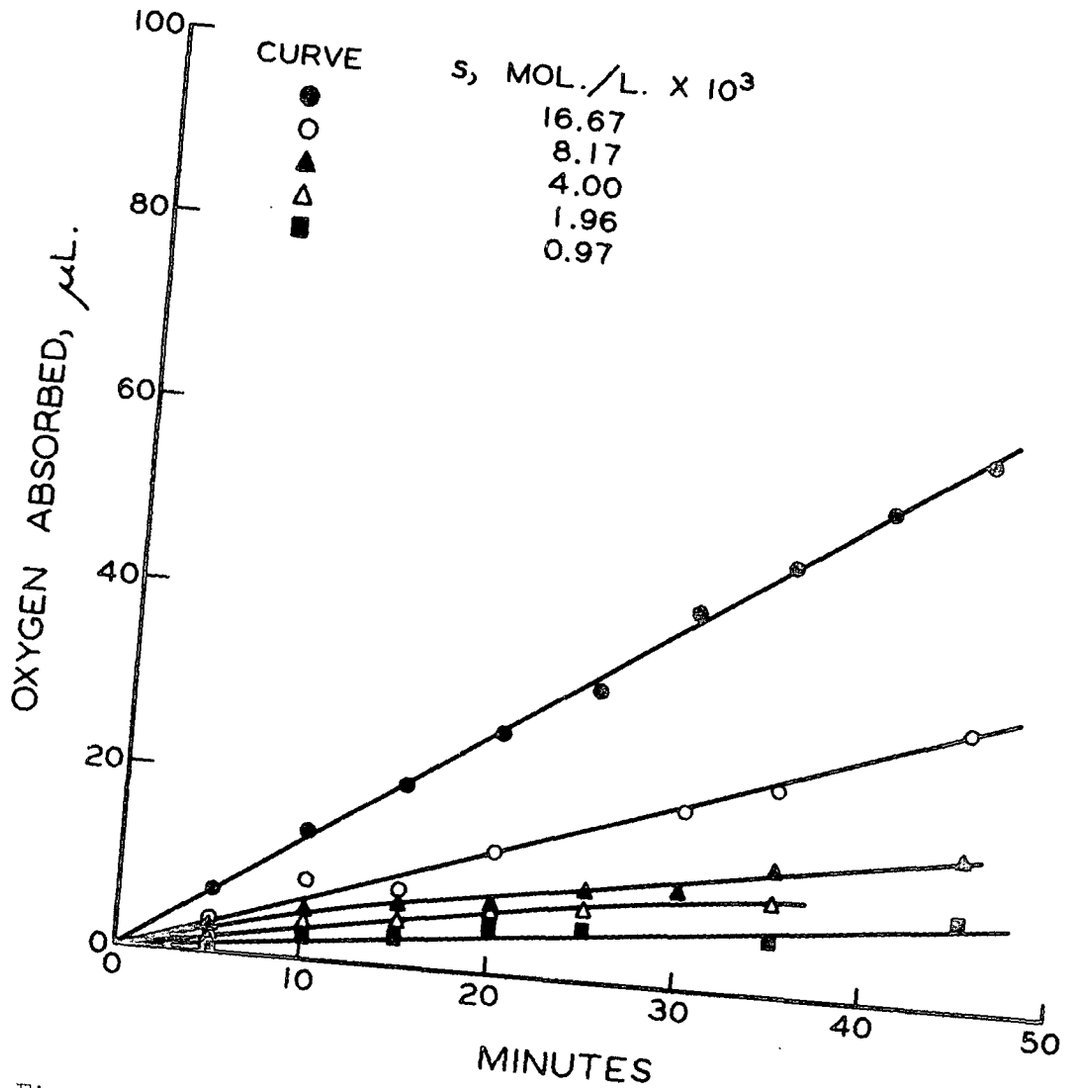


Figure 40. Oxygen Absorption-Time Curves for the p-Coumaryl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.7, at 25°C.

TABLE XXIV

REACTION RATE DATA FOR THE p-COUMARYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.7, AT 25°C. AS OBTAINED BY OXYGEN ABSORPTION MEASUREMENTS USING A WARBURG CONSTANT VOLUME RESPIROMETER

Substrate Concentration, s, moles/liter x 10^{3}	1/s, liters/mole x 10^{-3}	Time, minutes	Oxygen Absorbed, µl.[a]	Initial Rates: Oxygen Used, µl./3 ml./minute	Initial Rates: p-Coumaryl Alcohol, moles/liter-minute x 10^{5}	1/v, p-coumaryl alcohol, liter-minutes/mole x 10^{-4}
16.67 (1)[b]	0.060	5	6.9	1.34	7.28	1.37
		10	13.9			
		15	19.7			
		20	26.3			
		25	31.9			
		30	41.0			
		35	46.9			
		40	53.3			
		45	59.5			
8.17 (2)	0.122	5	3.4	0.67	3.64	2.82
		10	8.3			
		15	8.2			
		20	13.1			
		30	19.4			
		35	22.3			
		45	30.3			
4.00 (3)	0.250	5	2.5	0.52	2.86	3.5
		10	5.3			
		15	6.7			
		20	7.6			
		25	9.9			
		30	10.6			
		35	13.8			
		45	16.3			
1.96 (4)	0.510	5	1.7	0.33	1.82	5.5
		10	3.9			
		15	4.6			
		20	6.8			
		25	7.8			
		35	9.6			
0.97 (5)	1.033	5	0.8	0.20	1.08	9.3
		10	2.4			
		15	2.7			
		20	4.7			
		25	5.5			
		35	5.9			
		45	9.4			

[a] Average of three determinations.

[b] Number in parentheses refers to the respective curve in Fig. 40.

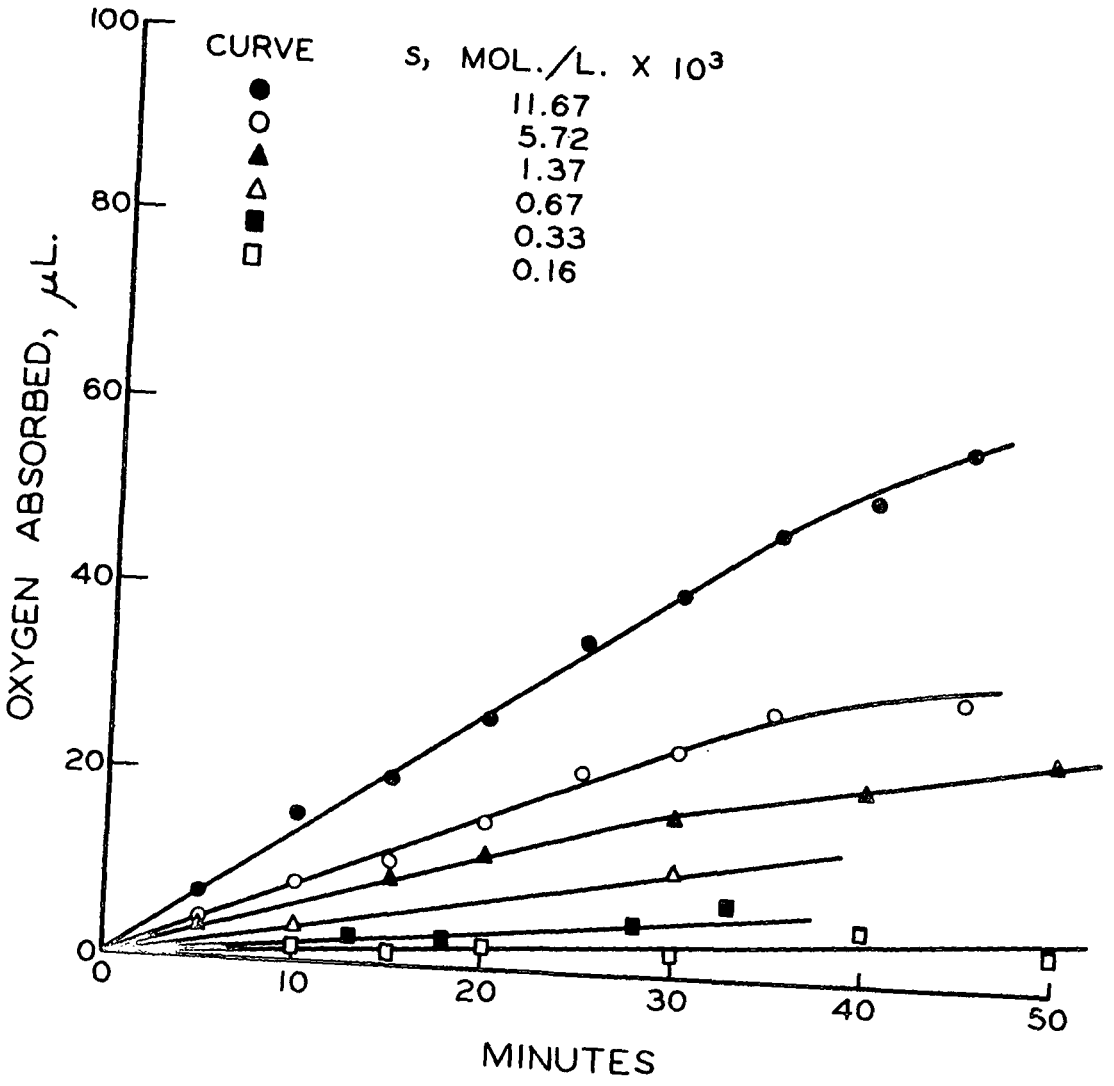


Figure 41. Oxygen Absorption-Time Curves for the p-Coumaryl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.2, at 25°C.

TABLE XXV

REACTION RATE DATA FOR THE p-COUMARYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.2, AT 25°C. AS OBTAINED BY OXYGEN ABSORPTION MEASUREMENTS USING A WARBURG CONSTANT VOLUME RESPIROMETER

Substrate Concentration, s, moles/liter x 10^3	1/s, liters/mole x 10^{-3}	Time, minutes	Oxygen Absorbed, µl.[a]	Initial Rates: Oxygen Used, µl./3 ml./minute	Initial Rates: p-Coumaryl Alcohol, moles/liter-minute x 10^5	1/v, p-coumaryl alcohol, liter-minutes/mole x 10^{-4}
11.67 (1)[b]	0.086	5	6.4	1.35	7.32	1.37
		10	15.2			
		15	19.7			
		20	26.7			
		25	35.2			
		30	40.8			
		35	47.9			
		40	51.3			
		45	57.6			
5.72 (2)	0.175	5	3.9	0.82	4.47	2.24
		10	8.3			
		15	10.8			
		20	15.2			
		25	21.3			
		30	23.6			
		35	28.5			
		45	30.1			
1.37 (3)	0.730	5	3.2	0.59	3.21	3.12
		15	9.1			
		20	12.0			
		30	16.6			
		40	20.2			
		50	24.3			
0.67 (4)	1.490	10	3.6	0.36	1.96	5.11
		30	10.8			
0.33 (5)	3.030	13	2.4	0.19	1.03	9.70
		18	3.9			
		28	5.2			
		33	7.8			
0.16 (6)	6.250	10	1.3	0.10	0.54	18.50
		15	1.1			
		20	2.2			
		30	2.0			
		40	5.3			
		50	3.5			

[a] Average of three determinations.

[b] Number in parentheses refers to the respective curve in Fig. 41.

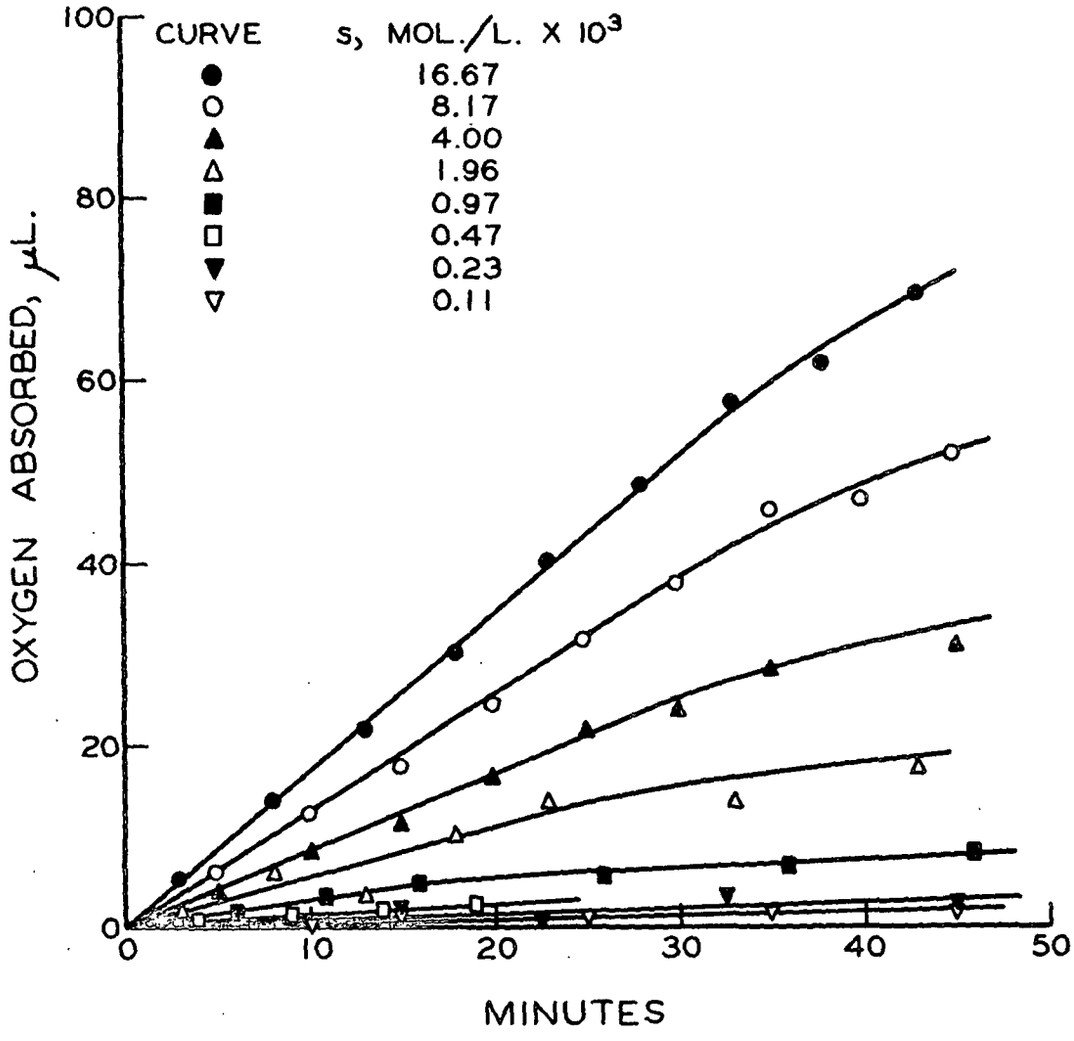


Figure 42. Oxygen Absorption-Time Curves for the p-Coumaryl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.7, at 25°C.

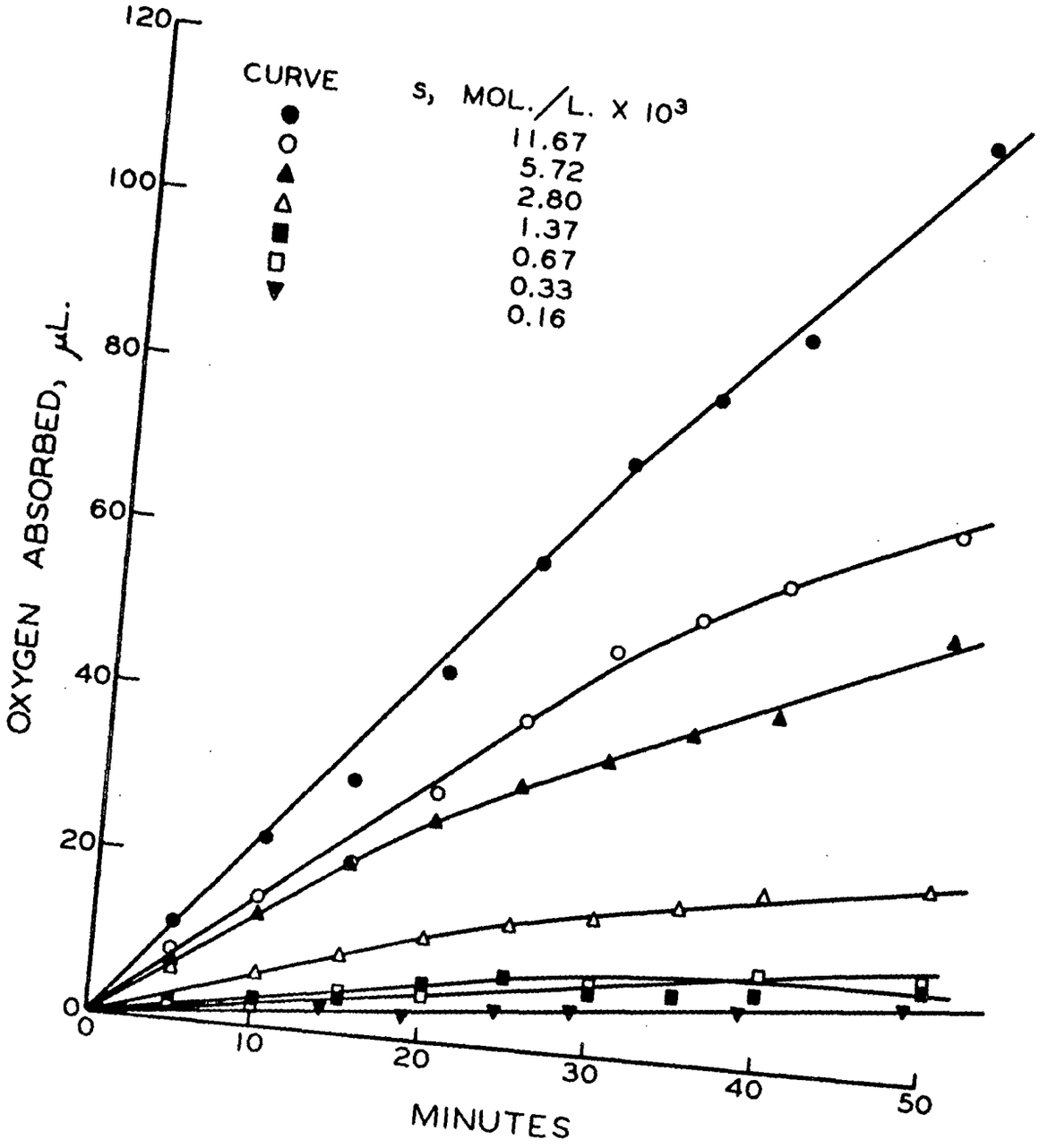


Figure 43. Oxygen Absorption-Time Curves for the Sinapyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.2, at 25°C.

TABLE XXVII

REACTION RATE DATA FOR THE SINAPYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.2, AT 25°C. AS OBTAINED BY OXYGEN ABSORPTION MEASUREMENTS USING A WARBURG CONSTANT VOLUME RESPIROMETER

Substrate Concentration, s, moles/liter x 10^{3}	1/s, liters/mole x 10^{-3}	Time, minutes	Oxygen Absorbed, µl.[a]	Initial Rates: Oxygen Used, µl./3 ml./minute	Initial Rates: Sinapyl Alcohol, moles/liter-minute x 10^{5}	1/v, sinapyl alcohol, liter-minutes/mole x 10^{-4}
11.67 (1)[b]	0.086	5	11.4	2.36	12.88	0.78
		10	22.4			
		15	30.4			
		20	44.8			
		25	58.9			
		30	71.4			
		40	88.7			
5.72 (2)	0.175	5	8.3	1.59	8.64	1.16
		10	15.2			
		15	20.2			
		20	30.0			
		25	39.3			
		30	49.0			
		40	58.7			
2.80 (3)	0.357	5	6.8	1.38	7.52	1.33
		10	13.5			
		15	20.4			
		20	26.3			
		25	31.6			
		30	35.4			
		40	42.9			
1.37 (4)	0.730	10	6.2	0.63	3.04	3.28
		15	9.2			
		20	12.2			
		25	14.6			
		30	16.3			
		40	20.8			
0.67 (5)	1.490	5	1.7	0.33	1.80	5.55
		10	2.9			
		15	4.0			
		20	6.8			
		25	8.2			
		30	7.2			
		40	8.8			
0.33 (6)	3.030	5	2.0	0.28	1.50	6.67
		10	2.5			
		15	4.1			
		20	5.6			
		30	8.3			
		40	11.1			
0.16 (7)	6.250	14	2.9	0.18	0.96	10.4
		19	2.7			
		24	4.4			
		29	5.1			
		39	6.9			
		49	8.8			

[a] Average of three determinations.

[b] Number in parentheses refers to the respective curve in Fig. 43.

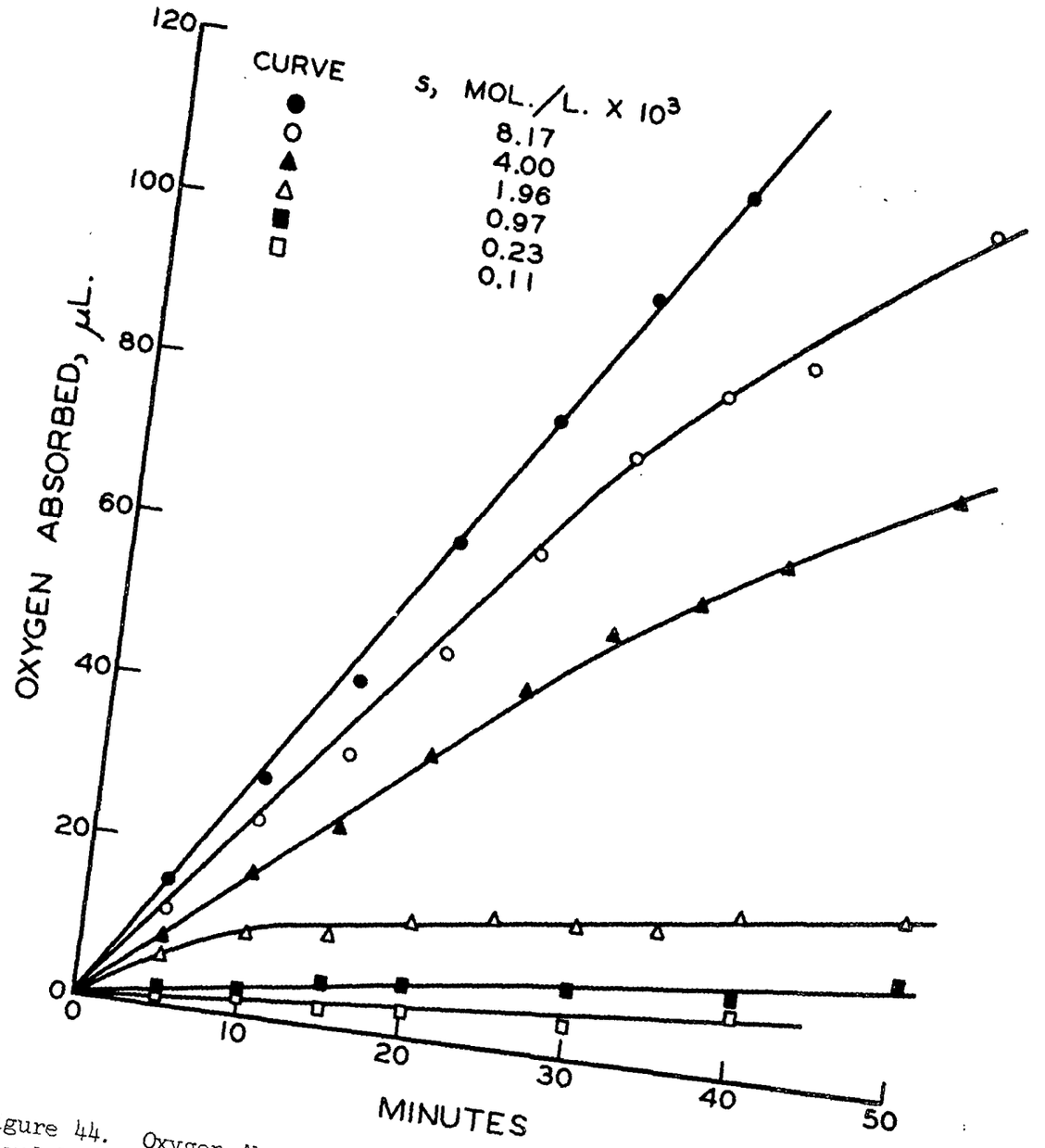


Figure 44. Oxygen Absorption-Time Curves for the Sinapyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.7, at 25°C.

TABLE XXVIII

REACTION RATE DATA FOR THE SINAPYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.7, AT 25°C. AS OBTAINED BY OXYGEN ABSORPTION MEASUREMENTS USING A WARBURG CONSTANT VOLUME RESPIROMETER

Substrate Concentration, s, moles/liter x 10^3	$1/s$, liters/mole x 10^{-3}	Time, minutes	Oxygen Absorbed,[a] µl.	Initial Rates: Oxygen Used, µl./3 ml./minute	Initial Rates: Sinapyl Alcohol, moles/liter-minute x 10^5	$1/v$, sinapyl alcohol, liter-minutes/mole x 10^{-4}
8.17 (1)[b]	0.122	5	15.4	3.10	16.80	0.60
		10	29.1			
		15	42.9			
		20	61.1			
		25	77.5			
		30	94.2			
		35	107.9			
4.00 (2)	0.250	5	11.8	2.50	13.56	0.74
		10	24.0			
		15	33.5			
		20	47.5			
		25	61.0			
		30	74.4			
		35	83.5			
		40	88.4			
		50	107.6			
1.96 (3)	0.510	5	8.2	1.73	9.44	1.06
		10	17.5			
		15	24.1			
		20	34.9			
		25	44.2			
		30	52.4			
		35	57.5			
		40	63.5			
		50	74.2			
0.97 (4)	1.033	5	6.1	1.25	6.85	1.46
		10	9.8			
		15	11.0			
		20	13.7			
		25	15.5			
		30	16.0			
		35	16.9			
		40	19.6			
		50	22.2			
0.23 (5)	4.305	5	1.9	0.32	1.73	5.80
		10	2.7			
		15	5.0			
		20	6.1			
		30	8.0			
		40	10.6			
		50	12.4			
0.11 (6)	8.780	5	0.6	0.18	1.00	10.00
		10	2.0			
		15	1.8			
		20	3.8			
		30	4.1			
		40	7.9			

[a] Average of three determinations.

[b] Number in parentheses refers to the respective curve in Fig. 44.

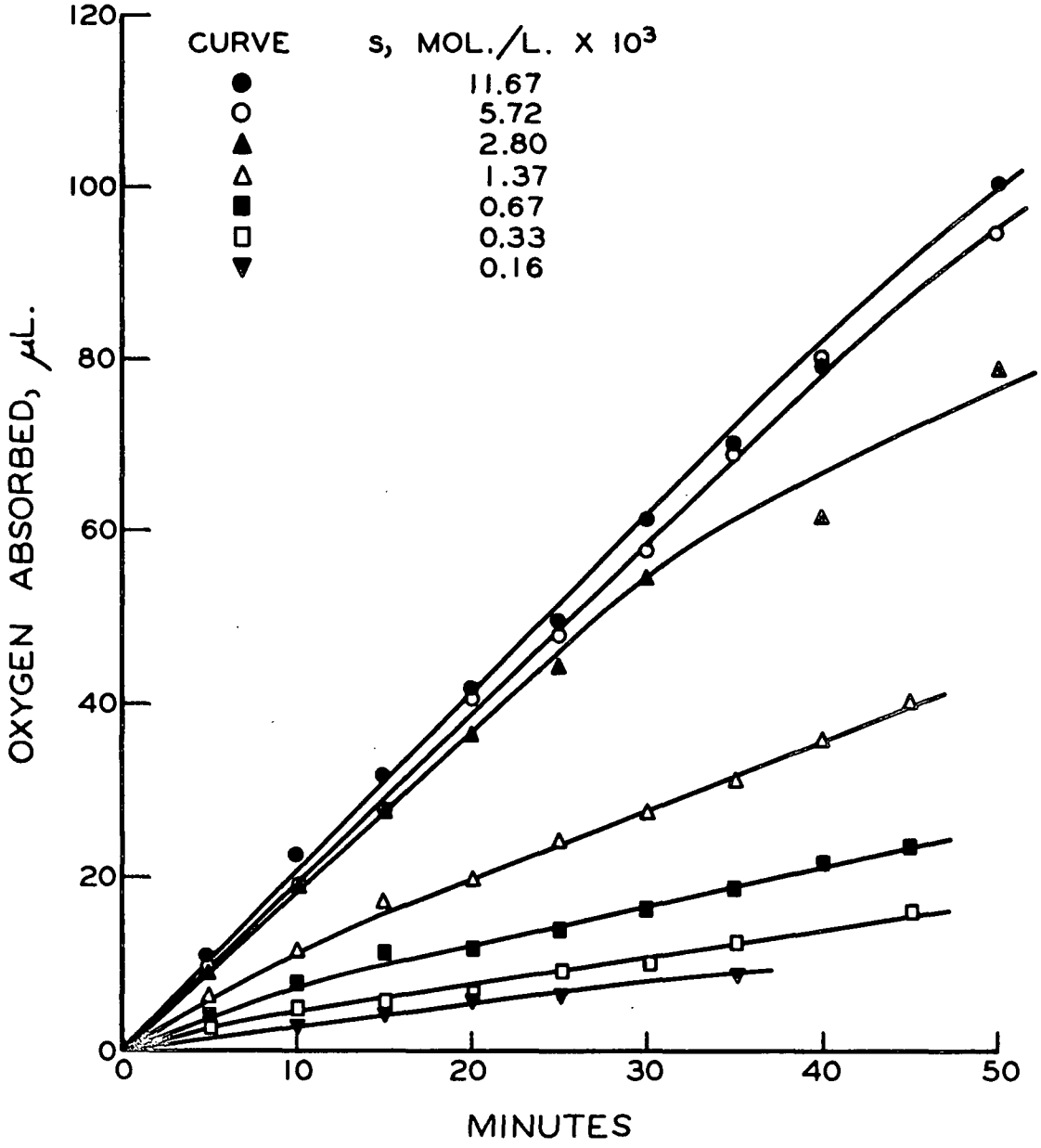


Figure 45. Oxygen Absorption-Time Curves for the Sinapyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.2, at 25°C.

TABLE XXIX

REACTION RATE DATA FOR THE SINAPYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.2, AT 25°C. AS OBTAINED BY OXYGEN ABSORPTION MEASUREMENTS USING A WARBURG CONSTANT VOLUME RESPIROMETER

Substrate Concentration, s, moles/liter x 10^{3}	$1/s$, liters/mole x 10^{-3}	Time, minutes	Oxygen Absorbed, µl.[a]	Initial Rates: Oxygen Used, µl./3 ml./minute	Initial Rates: Sinapyl Alcohol, moles/liter-minute x 10^{5}	$1/v$, sinapyl alcohol, liter-minutes/mole x 10^{-4}
11.67 (1)[b]	0.086	5	10.8	2.08	11.28	0.89
		10	22.5			
		15	31.7			
		20	41.8			
		30	61.1			
		40	79.3			
5.72 (2)	0.175	5	10.0	1.94	10.60	0.94
		10	19.1			
		15	28.0			
		20	40.4			
		25	48.2			
		30	57.9			
		40	79.5			
2.80 (3)	0.357	5	9.4	1.85	10.12	0.99
		10	19.2			
		15	28.1			
		20	36.6			
		25	44.3			
		30	54.8			
		40	61.5			
1.37 (4)	0.730	5	6.1	1.13	6.20	1.61
		10	11.4			
		15	17.2			
		20	19.6			
		25	24.3			
		35	31.2			
		45	40.2			
0.67 (5)	1.490	5	3.8	0.74	4.08	2.24
		10	7.6			
		15	11.1			
		20	11.5			
		25	13.9			
		35	18.6			
		45	23.4			
0.33 (6)	3.030	5	2.8	0.60	2.76	3.62
		10	4.6			
		15	5.3			
		20	6.5			
		25	9.2			
		35	12.3			
		45	12.9			
0.16 (7)	6.250	10	4.2	0.27	1.48	6.75
		15	4.4			
		20	5.6			
		25	6.1			
		35	8.5			

[a] Average of three determinations.

[b] Number in parentheses refers to the respective curve in Fig. 45.

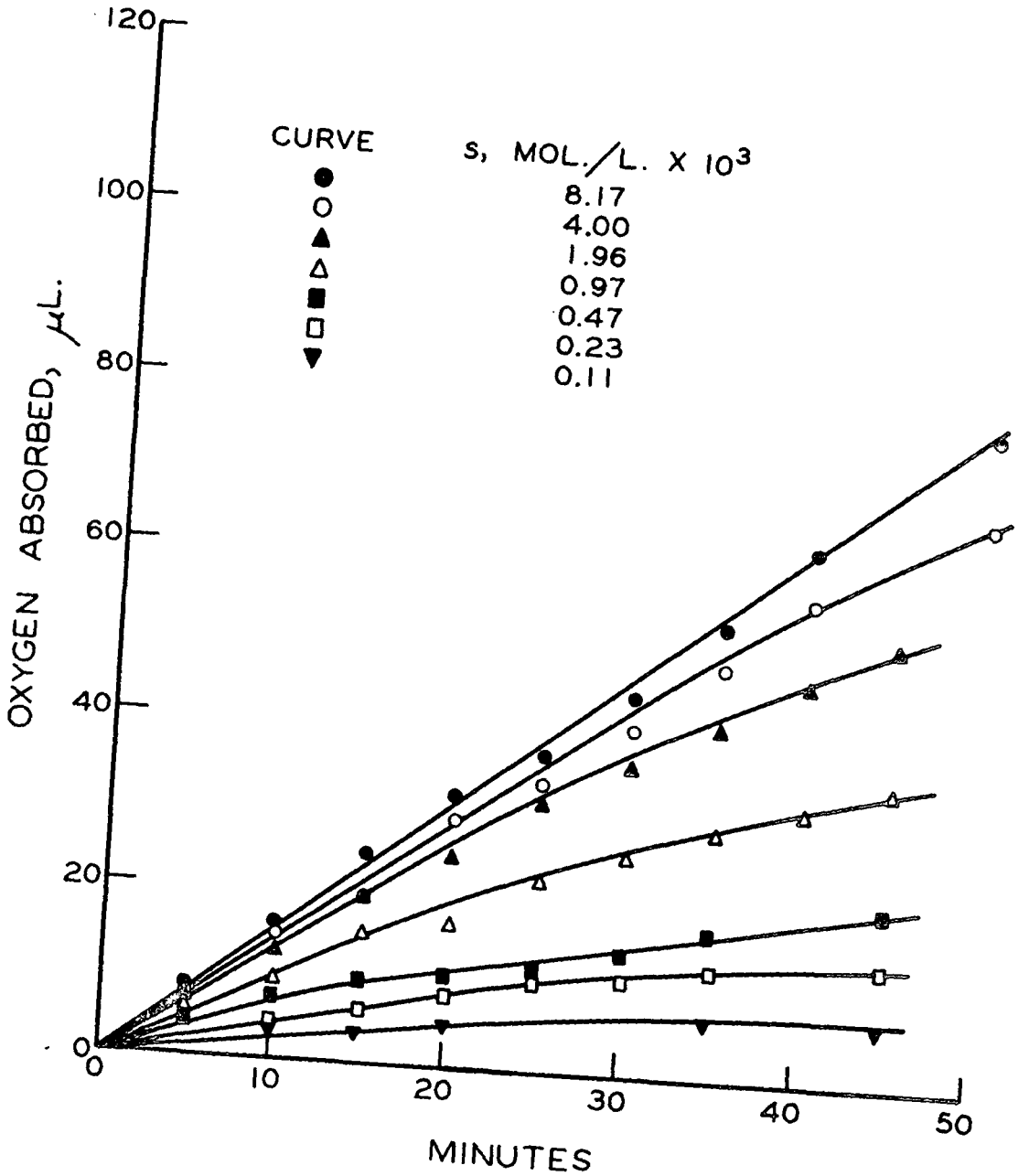


Figure 46. Oxygen Absorption-Time Curves for the Sinapyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.7, at 25°C.

TABLE XXX

REACTION RATE DATA FOR THE SINAPYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.7, AT 25°C. AS OBTAINED BY OXYGEN ABSORPTION MEASUREMENTS USING A WARBURG CONSTANT VOLUME RESPIROMETER

Substrate Concentration, s, moles/liter x 10^{3}	1/s, liters/mole x 10^{-3}	Time, minutes	Oxygen Absorbed,[a] µl.	Initial Rates: Oxygen Used, µl./3 ml./minute	Initial Rates: Sinapyl Alcohol, moles/liter-minute x 10^{5}	1/v, sinapyl alcohol, liter-minutes/mole x 10^{-4}
8.17 (1)[b]	0.122	5	8.3	1.57	8.48	1.18
		10	16.0			
		15	24.5			
		20	31.8			
		25	37.1			
		30	44.5			
		40	62.5			
4.00 (2)	0.250	5	7.1	1.44	7.84	1.28
		10	14.5			
		15	19.6			
		20	29.1			
		25	33.7			
		30	40.8			
		40	56.2			
1.96 (3)	0.510	5	6.4	1.24	6.44	1.55
		10	12.5			
		15	19.6			
		20	24.9			
		25	31.4			
		35	41.2			
		45	51.9			
0.97 (4)	1.033	5	5.3	1.00	5.40	1.85
		10	9.3			
		15	15.3			
		20	16.7			
		25	22.0			
		35	23.9			
		45	34.7			
0.47 (5)	2.118	5	3.8	0.69	3.76	2.66
		10	7.2			
		15	9.6			
		20	10.5			
		25	12.0			
		35	17.4			
		45	20.5			
0.23 (6)	4.305	10	4.3	0.43	2.28	4.39
		15	6.2			
		20	8.5			
		25	10.4			
		30	11.1			
		35	12.7			
		45	14.0			
0.11 (7)	8.780	10	3.2	0.24	1.28	7.81
		15	3.2			
		20	4.9			
		35	7.0			
		45	6.8			

[a] Average of three determinations.

[b] Number in parentheses refers to the respective curve in Fig. 46.

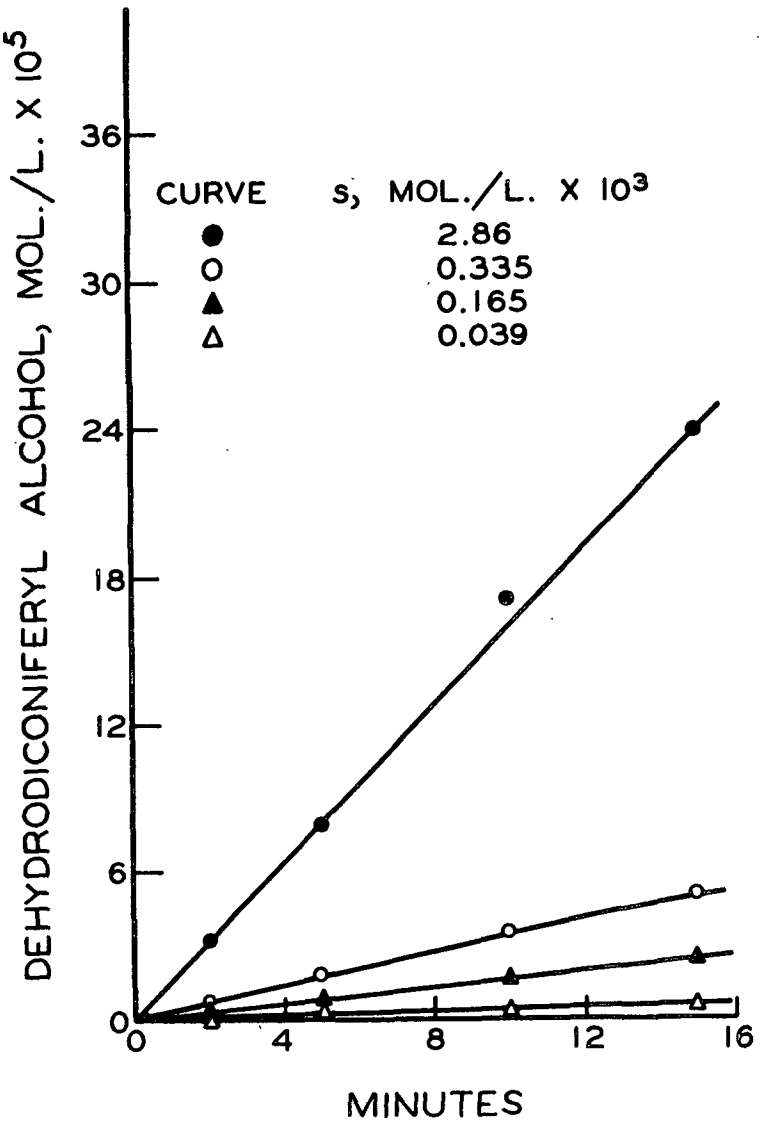


Figure 47. Moles Dehydrodiconiferyl Alcohol Occurring with Time in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.2

TABLE XXXI

REACTION RATE DATA FOR THE OCCURRENCE OF DEHYDRODICONIFERYL ALCOHOL IN THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.2, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^3	$1/s$, liters/mole x 10^{-3}	Time, minutes	Dehydrodiconiferyl Alcohol, moles/liter x 10^5	Initial Rates: Dehydrodiconiferyl Alcohol, moles/liter-min. x 10^6	$1/v$, dehydrodiconiferyl alcohol, liter-min./mole x 10^{-5}
2.86 (1)[a]	0.350	2	3.20	16.0	0.63
		5	7.95		
		10	17.05		
		15	23.95		
0.335 (2)	2.980	2	0.74	3.48	2.88
		5	1.88		
		10	3.59		
		15	5.15		
0.165 (3)	6.060	2	0.39	1.67	6.0
		5	0.93		
		10	1.76		
		15	2.64		
0.039 (4)	25.640	2	0.09	0.40	25.0
		5	0.29		
		10	0.45		
		15	0.67		

[a]Number in parentheses refers to the respective curve in Fig. 47.

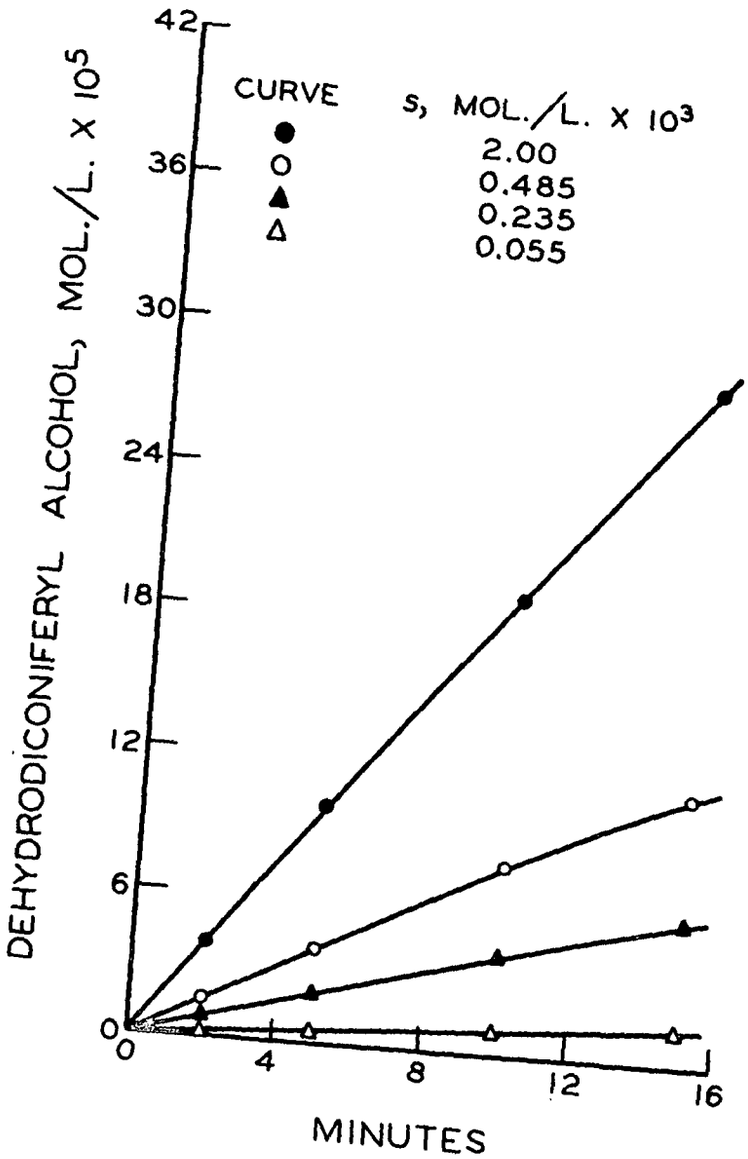


Figure 48. Moles Dehydrodiconiferyl Alcohol Occurring with Time in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.7

TABLE XXXII

REACTION RATE DATA FOR THE OCCURRENCE OF DEHYDRODICONIFERYL ALCOHOL IN THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.7, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^{3}	$1/s$, liters/mole x 10^{-3}	Time, minutes	Dehydrodiconiferyl Alcohol, moles/liter x 10^{5}	Initial Rates: Dehydrodiconiferyl Alcohol, moles/liter-min. x 10^{6}	$1/v$, dehydrodiconiferyl alcohol, liter-min./mole x 10^{-5}
2.00 (1)[a]	0.500	2	4.0	19.33	0.52
		5	9.8		
		10	19.1		
		15	28.2		
0.485 (2)	2.067	2	1.56	7.82	1.29
		5	3.99		
		10	7.80		
		15	11.20		
0.235 (3)	4.236	2	0.88	4.05	2.47
		5	2.15		
		10	4.18		
		15	6.10		
0.055 (4)	17.560	2	0.22	1.03	9.7
		5	0.55		
		10	1.05		
		15	1.54		

[a]Number in parentheses refers to the respective curve in Fig. 48.

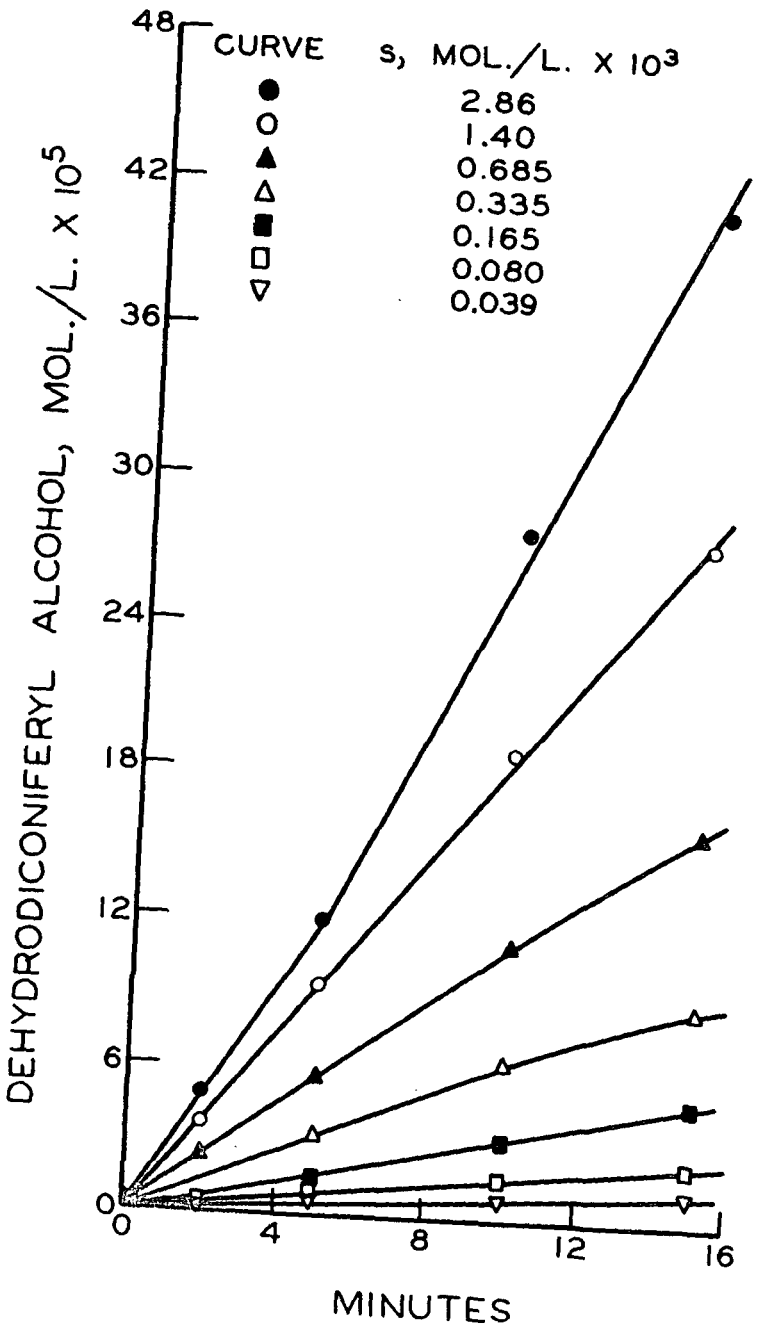


Figure 49. Moles Dehydrodiconiferyl Alcohol Occurring with Time in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.2

TABLE XXXIII

REACTION RATE DATA FOR THE OCCURRENCE OF DEHYDRODICONIFERYL ALCOHOL IN THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.2, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^3	$1/s$, liters/mole x 10^{-3}	Time, minutes	Dehydrodiconiferyl Alcohol, moles/liter x 10^5	Initial Rates Dehydrodiconiferyl Alcohol, moles/liter-min. x 10^6	$1/v$, dehydrodiconiferyl alcohol, liter-min./mole x 10^{-5}
2.86 (1)[a]	0.350	2	4.8	23.7	0.42
		5	11.9		
		10	26.4		
		15	41.3		
1.40 (2)	0.714	2	3.6	18.7	0.54
		5	9.4		
		10	19.0		
		15	27.7		
0.685 (3)	1.460	2	2.3	10.95	0.91
		5	5.6		
		10	11.1		
		15	15.9		
0.335 (4)	2.980	2	--	6.2	1.61
		5	3.2		
		10	6.4		
		15	8.8		
0.165 (5)	6.060	2	--	3.15	3.18
		5	1.6		
		10	3.2		
		15	4.9		
0.080 (6)	12.500	2	0.35	1.60	6.25
		5	0.90		
		10	1.67		
		15	2.44		
0.039 (7)	25.640	2	0.17	0.85	11.77
		5	0.45		
		10	0.87		
		15	1.28		

[a]Number in parentheses refers to the respective curve in Fig. 49.

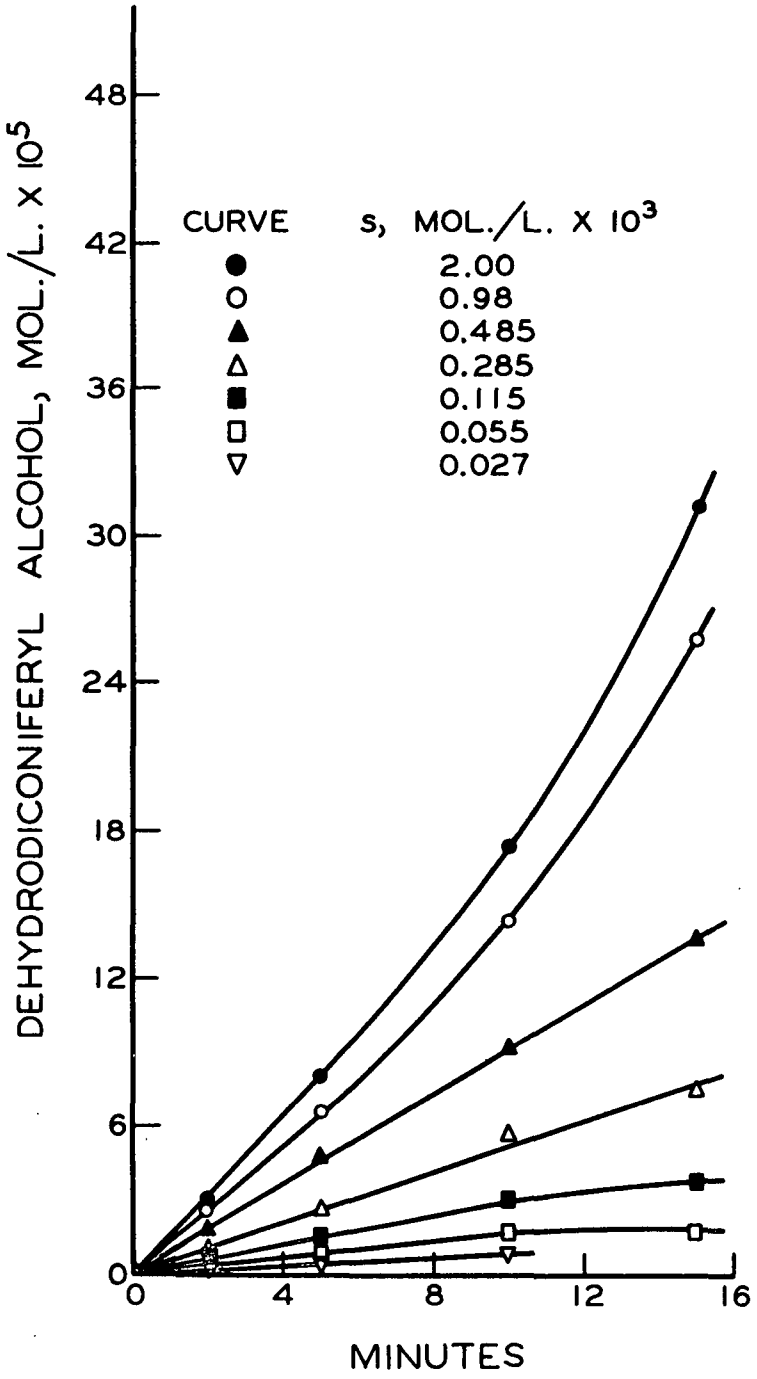


Figure 50. Moles Dehydrodiconiferyl Alcohol Occurring with Time in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.7

TABLE XXXIV

REACTION RATE DATA FOR THE OCCURRENCE OF DEHYDRODICONIFERYL ALCOHOL IN THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.7, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^{3}	$1/s$, liters/mole x 10^{-5}	Time, minutes	Dehydrodiconiferyl Alcohol, moles/liter x 10^{5}	Initial Rates Dehydrodiconiferyl Alcohol, moles/liter-min. x 10^{6}	$1/v$, dehydrodiconiferyl alcohol, liter-min / mole x 10^{-5}
2.000 (1)[a]	0.500	2	3.1	16.2	0.62
		5	8.1		
		10	17.4		
		15	25.3		
0.980 (2)	1.020	2	2.66	13.0	0.77
		5	6.64		
		10	14.30		
		15	19.90		
0.485 (3)	2.067	2	1.91	9.15	1.08
		5	4.85		
		10	9.31		
		15	13.70		
0.285 (4)	4.236	2	1.08	5.2	1.92
		5	2.70		
		10	5.80		
		15	7.60		
0.115 (5)	8.610	2	0.61	3.0	3.33
		5	1.59		
		10	3.13		
		15	3.88		
0.055 (6)	17.560	2	0.34	1.66	6.00
		5	0.85		
		10	1.74		
		15	1.75		
0.027 (7)	36.700	2	0.20	0.90	11.00
		5	0.46		
		10	0.93		
		15	--		

[a]Number in parentheses refers to the respective curve in Fig. 50.

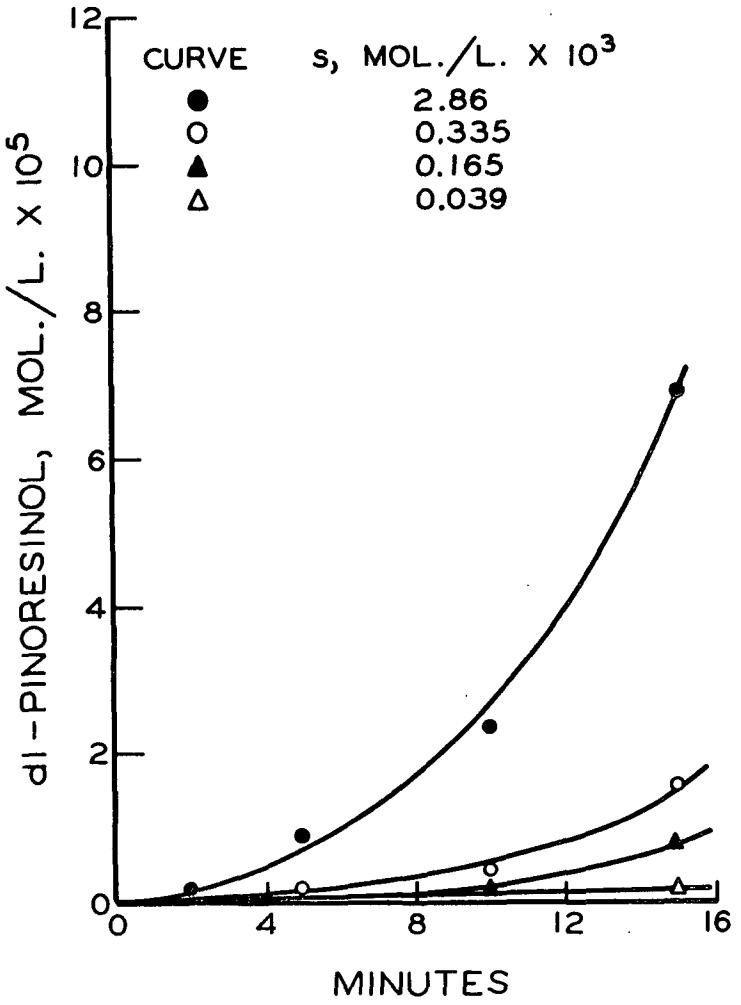


Figure 51. Moles dl-Pinoresinol Occurring with Time in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.2

TABLE XXXV

REACTION RATE DATA FOR THE OCCURRENCE OF dl-PINORESINOL IN THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.2, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^3	1/s, liters/mole x 10^{-3}	Time, minutes	dl-Pinoresinol, moles/liter x 10^5	Initial Rates dl-Pinoresinol, moles/liter-min. x 10^6	1/v, dl-pinoresinol, liter-minutes/mole x 10^{-5}
2.86 (1)[a]	0.350	2	0.19	0.90	11.0
		5	0.95		
		10	2.40		
		15	6.95		
0.335 (2)	2.980	2	--	0.30	33.3
		5	0.20		
		10	0.45		
		15	1.58		
0.165 (3)	6.060	2	--	0.18	55.6
		5	--		
		10	0.23		
		15	0.84		
0.039 (4)	25.640	2	--	0.05	200.0
		5	--		
		10	--		
		15	0.20		

[a]Number in parentheses refers to the respective curve in Fig. 51.

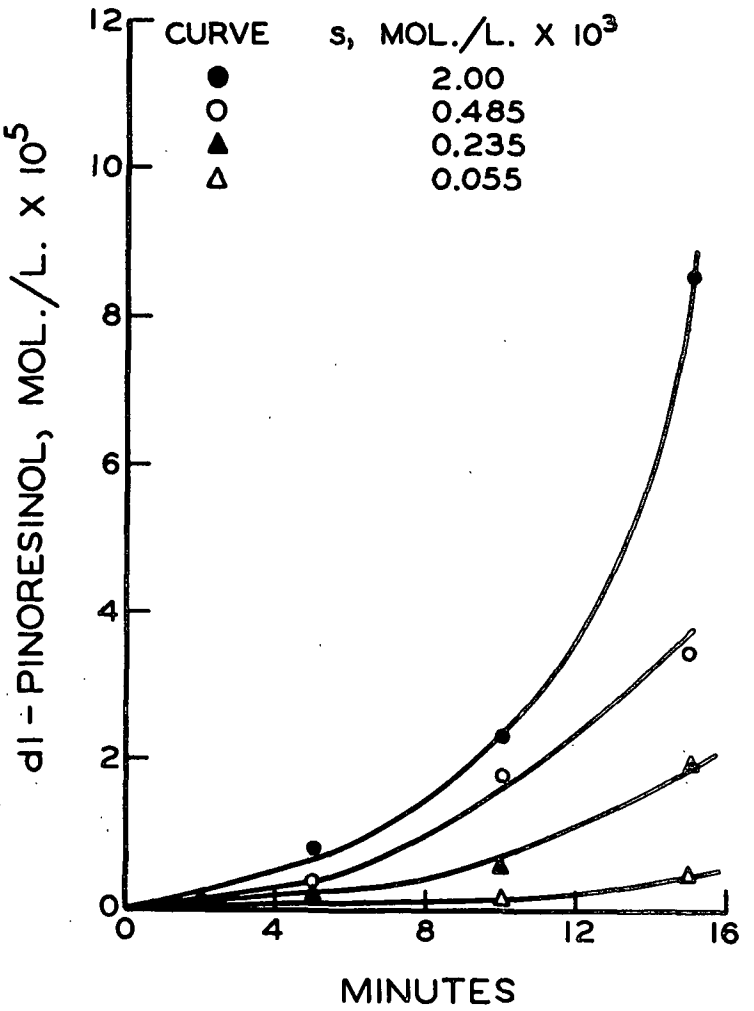


Figure 52. Moles dl-Pinoresinol Occurring with Time in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.7

TABLE XXXVI

REACTION RATE DATA FOR THE OCCURRENCE OF dl-PINORESINOL IN THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.7, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^{3}	1/s, liters/mole x 10^{-3}	Time, minutes	dl-Pinoresinol, moles/liter x 10^{5}	Initial Rates dl-Pinoresinol, moles/liter-min. x 10^{6}	1/v, dl-pinoresinol, liter-minutes/mole x 10^{-5}
2.00 (1)[a]	0.500	2	0.22	1.30	7.7
		5	0.82		
		10	2.38		
		15	8.60		
0.485 (2)	2.067	2	--	0.69	14.50
		5	0.37		
		10	1.05		
		15	3.47		
0.235 (3)	4.236	2	--	0.46	21.8
		5	0.21		
		10	0.60		
		15	1.98		
0.055 (4)	17.560	2	--	0.14	71.4
		5	--		
		10	0.16		
		15	0.50		

[a]Number in parentheses refers to the respective curve in Fig. 52.

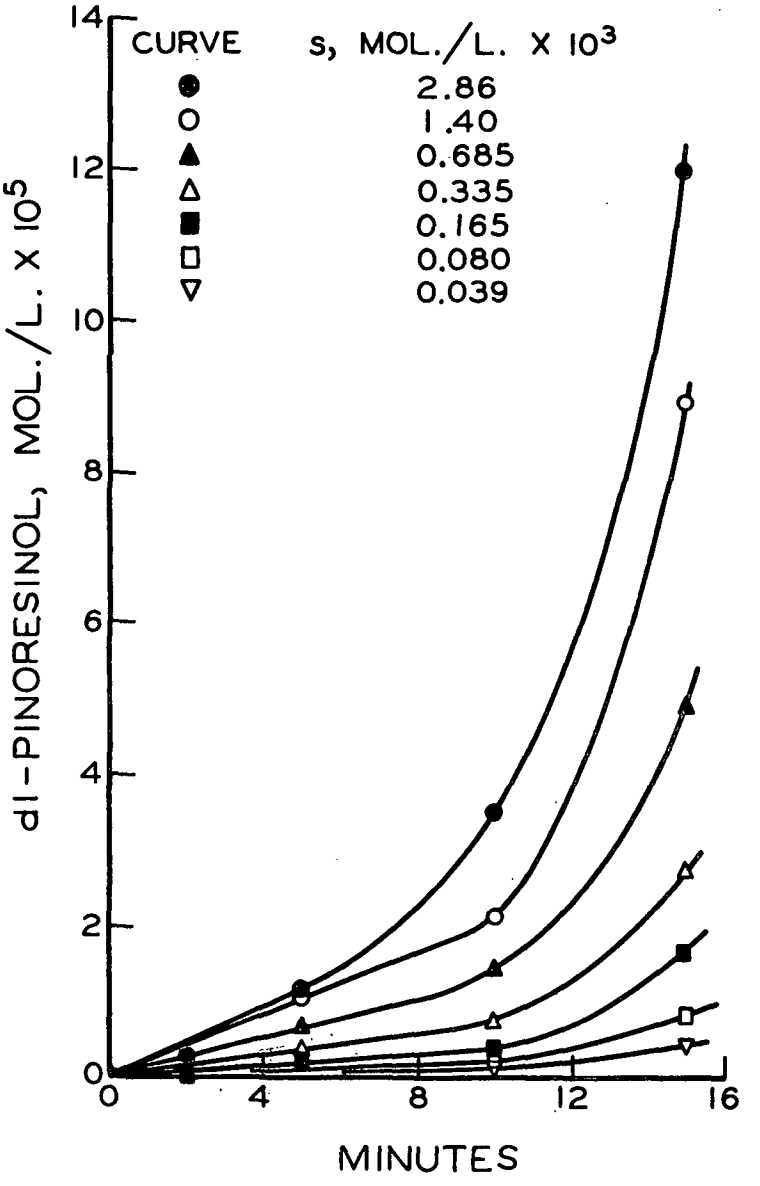


Figure 53. Moles dl-Pinoresinol Occurring with Time in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.2

TABLE XXXVII

REACTION RATE DATA FOR THE OCCURRENCE OF dl-PINORESINOL IN THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.2, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^3	1/s, liters/mole x 10^{-3}	Time, minutes	dl-Pinoresinol, moles/liter x 10^5	Initial Rates dl-Pinoresinol, moles/liter-min. x 10^6	1/v, dl-pinoresinol, liter-minutes/mole x 10^{-5}
2.86 (1)[a]	0.350	2	0.30	2.29	4.36
		5	1.20		
		10	3.50		
		15	12.00		
1.40 (2)	0.714	2	0.19	2.03	4.93
		5	1.06		
		10	2.10		
		15	8.90		
0.685 (3)	1.460	2	0.12	1.30	7.69
		5	0.66		
		10	1.44		
		15	4.90		
0.335 (4)	2.980	2	--	0.72	13.90
		5	0.39		
		10	0.74		
		15	2.70		
0.165 (5)	6.060	2	0.04	0.36	27.80
		5	0.18		
		10	0.39		
		15	1.65		
0.080 (6)	12.500	2	--	0.20	50.0
		5	--		
		10	0.21		
		15	0.80		
0.039 (7)	25.640	2	--	0.11	90.0
		5	--		
		10	0.11		
		15	0.43		

[a]Number in parentheses refers to the respective curve in Fig. 53.

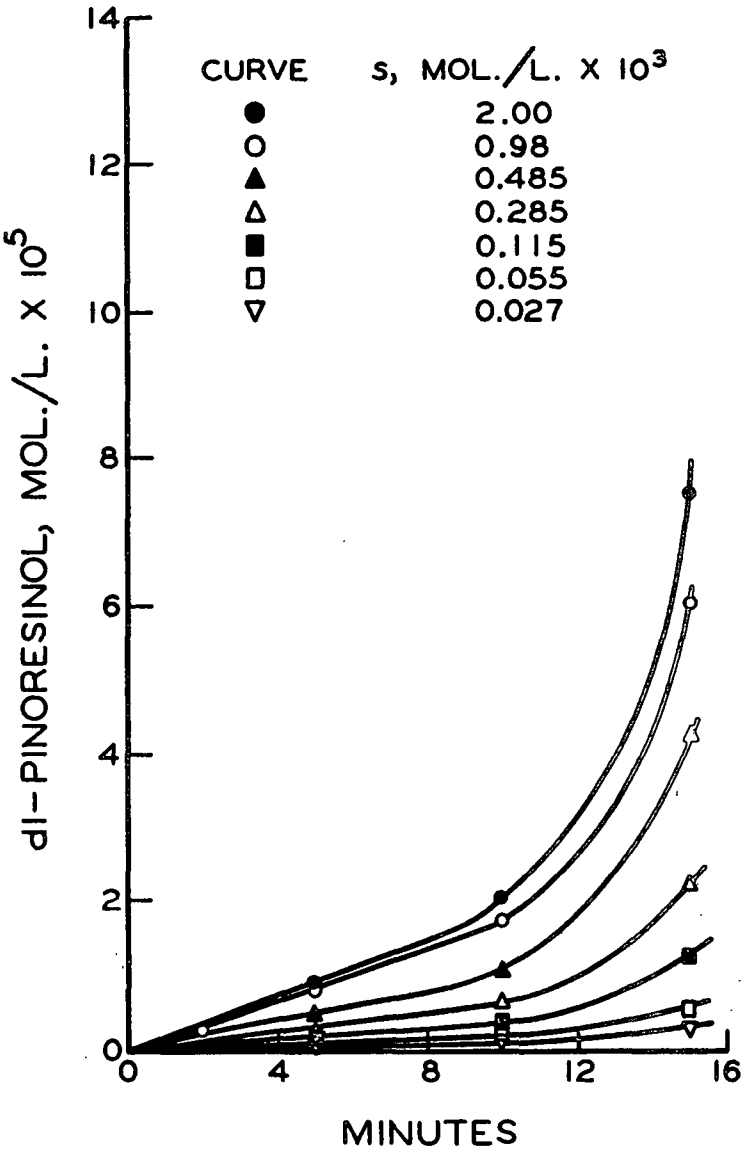


Figure 54. Moles dl-Pinoresinol Occurring with Time in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.7

TABLE XXXVIII

REACTION RATE DATA FOR THE OCCURRENCE OF dl-PINORESINOL IN THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.7, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^3	1/s, liters/mole x 10^{-3}	Time, minutes	dl-Pinoresinol, moles/liter x 10^5	Initial Rates dl-Pinoresinol, moles/liter-min. x 10^6	1/v, dl-pinoresinol, liter-minutes/mole x 10^{-5}
2.00 (1)[a]	0.500	2	0.20	1.85	5.41
		5	0.90		
		10	2.08		
		15	7.58		
0.98 (2)	1.020	2	0.27	1.73	5.78
		5	0.88		
		10	1.76		
		15	6.05		
0.485 (3)	2.067	2	0.26	0.98	10.21
		5	0.47		
		10	1.08		
		15	4.30		
0.285 (4)	4.236	2	--	0.63	15.88
		5	0.22		
		10	0.68		
		15	2.24		
0.115 (5)	8.610	2	--	0.36	27.80
		5	0.12		
		10	0.39		
		15	1.27		
0.055 (6)	17.560	2	--	0.19	52.60
		5	0.06		
		10	0.20		
		15	0.56		
0.027 (7)	36.70	2	--	0.10	100.0
		5	0.03		
		10	0.12		
		15	0.30		

[a]Number in parentheses refers to the respective curve in Fig. 54.

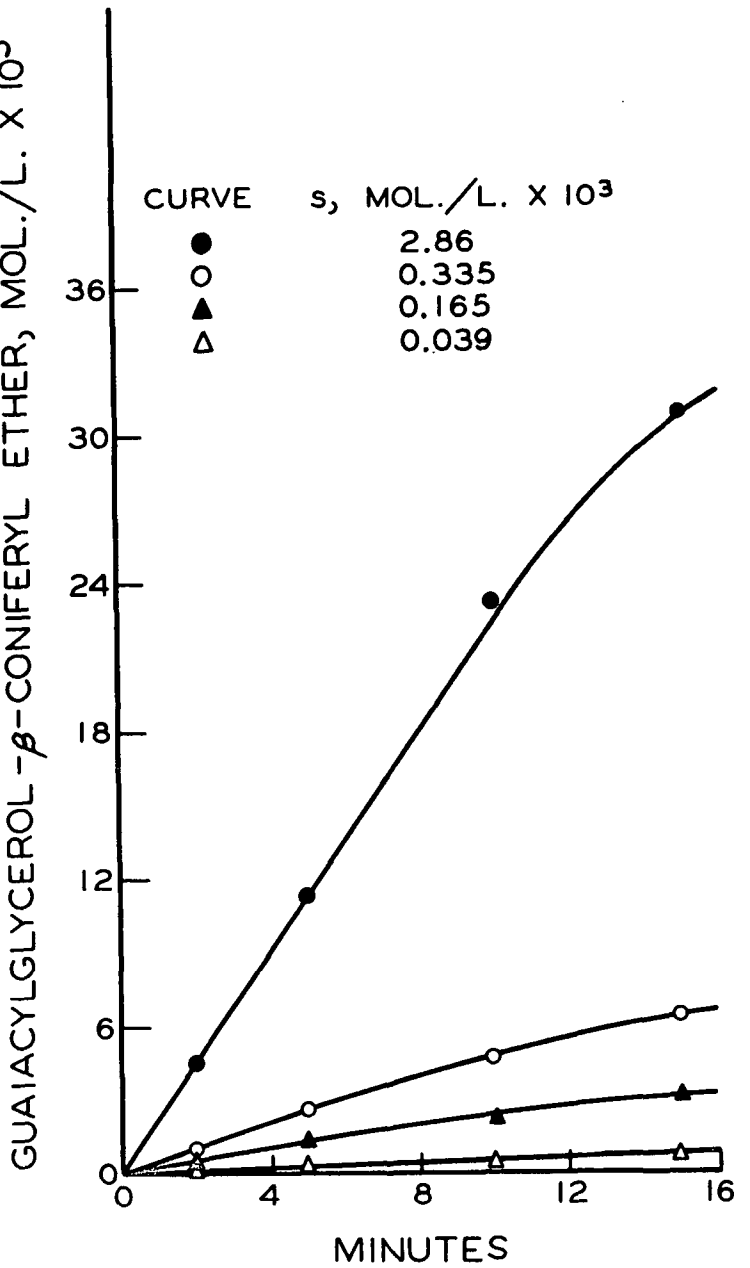


Figure 55. Moles Guaiacylglycerol-β-coniferyl Ether Occurring with Time in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.2

TABLE XXXIX

REACTION RATE DATA FOR THE OCCURRENCE OF GUAIACYLGLYCEROL-β-CONIFERYL ETHER IN THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.2, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, $\underline{s}$, moles/liter x 10^3	$1/\underline{s}$, liters/mole x 10^{-3}	Time, minutes	Guaiacylglycerol-β-Coniferyl Ether, moles/liter x 10^5	Initial Rates Guaiacylglycerol-β-Coniferyl Ether, moles/liter-min. x 10^6	$1/\underline{v}$, guaiacylglycerol-β-coniferyl ether, liter-min./mole x 10^{-5}
2.86 (1)[a]	0.350	2	4.55	22.4	0.45
		5	11.30		
		10	23.35		
		15	31.00		
0.335 (2)	2.980	2	1.04	5.15	1.94
		5	2.66		
		10	4.78		
		15	6.45		
0.165 (3)	6.060	2	0.55	2.5	4.0
		5	1.35		
		10	2.41		
		15	3.24		
0.039 (4)	25.40	2	0.14	0.63	15.90
		5	0.35		
		10	0.57		
		15	0.82		

[a]Number in parentheses refers to the respective curve in Fig. 55.

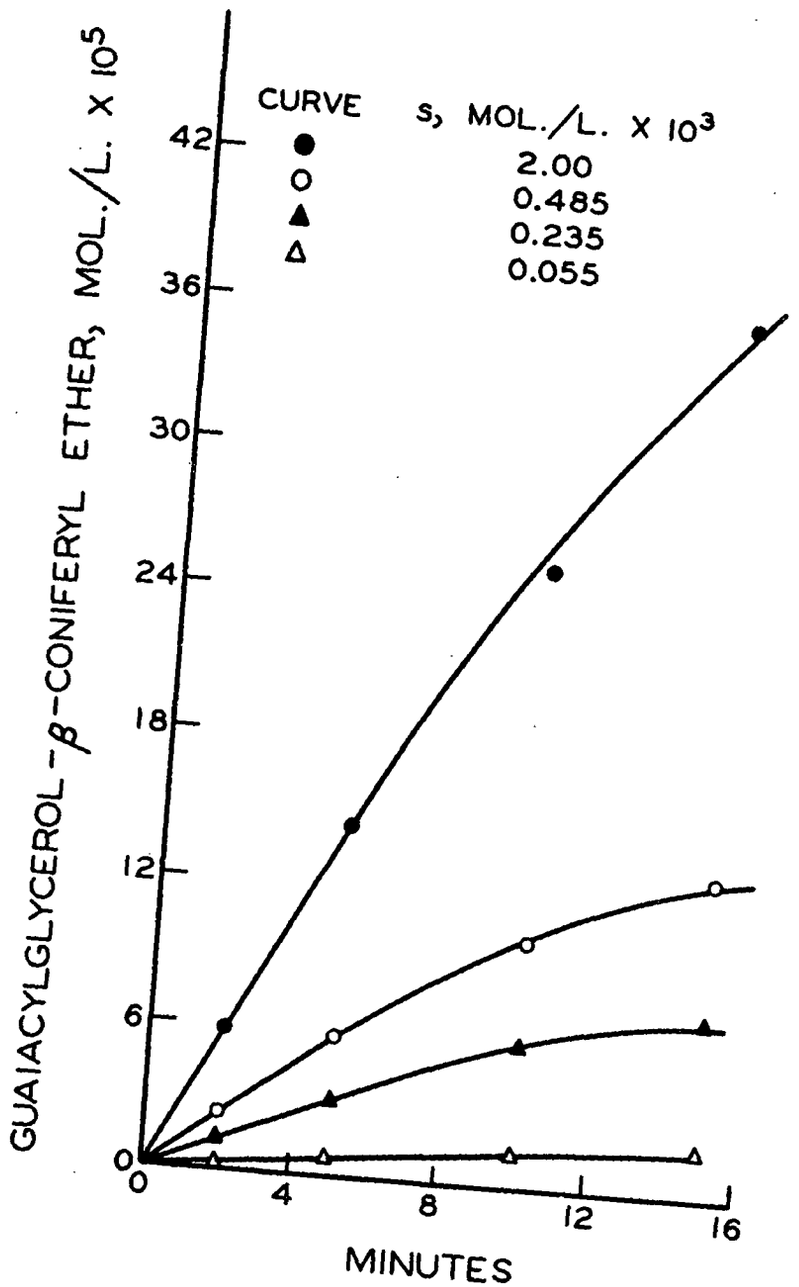


Figure 56. Moles Guaiacylglycerol-β-coniferyl Ether Occurring with Time in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 6.7

TABLE XL

REACTION RATE DATA FOR THE OCCURRENCE OF GUAIACYLGLYCEROL-β-CONIFERYL ETHER IN THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 6.7, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^3	$1/s$, liters/mole x 10^{-3}	Time, minutes	Guaiacylglycerol-β-Coniferyl Ether, moles/liter x 10^5	Initial Rates Guaiacylglycerol-β-Coniferyl Ether, moles/liter-min. x 10^6	$1/v$, guaiacyl-glycerol-β-coniferyl ether, liter-min./mole x 10^{-5}
2.00 (1)[a]	0.500	2	5.85	28.5	0.35
		5	14.50		
		10	25.40		
		15	35.85		
0.485 (2)	2.067	2	2.35	11.3	0.89
		5	5.80		
		10	10.05		
		15	13.00		
0.235 (3)	4.236	2	1.31	6.13	1.63
		5	3.16		
		10	5.85		
		15	7.30		
0.055 (4)	17.560	2	0.32	1.48	6.76
		5	0.80		
		10	1.41		
		15	1.92		

[a]Number in parentheses refers to the respective curve in Fig. 56.

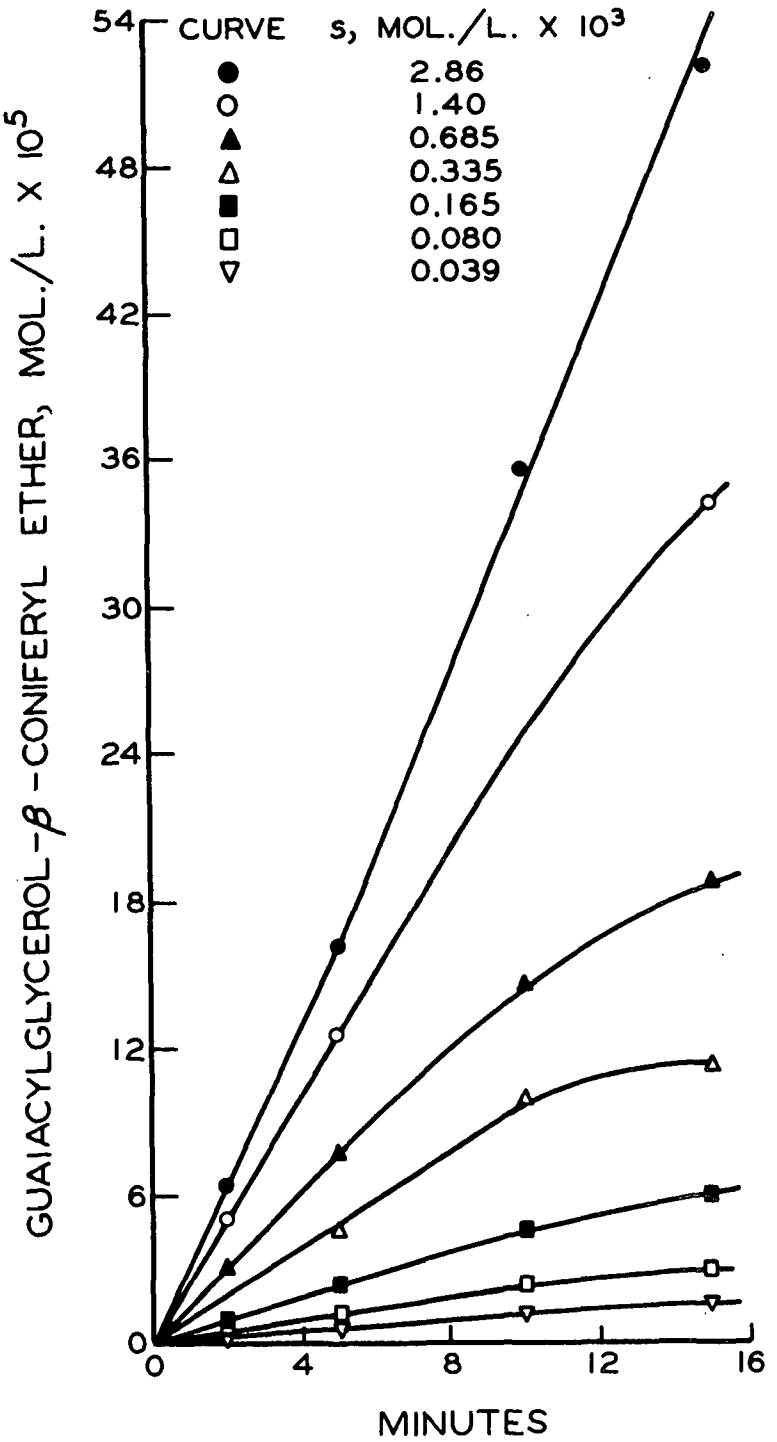


Figure 57. Moles Guaiacylglycerol-β-coniferyl Ether Occurring with Time in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.2

TABLE XLI

REACTION RATE DATA FOR THE OCCURRENCE OF GUAIACYLGLYCEROL-β-CONIFERYL ETHER IN THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.2, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, s, moles/liter x 10^3	$1/s$, liters/mole x 10^{-3}	Time, minutes	Guaiacylglycerol-β-Coniferyl Ether, moles/liter x 10^5	Initial Rates Guaiacylglycerol-β-Coniferyl Ether, moles/liter-min. x 10^6	$1/v$, guaiacylglycerol-β-coniferyl ether, liter-min./mole x 10^{-5}
2.86 (1)[a]	0.350	2	6.5	32.1	0.31
		5	16.3		
		10	35.6		
		15	52.1		
1.40 (2)	0.714	2	5.04	25.2	0.40
		5	12.50		
		10	--		
		15	34.20		
0.685 (3)	1.460	2	3.09	15.3	0.65
		5	7.75		
		10	14.60		
		15	18.80		
0.335 (4)	2.980	2	--	9.6	1.04
		5	4.5		
		10	8.5		
		15	11.3		
0.165 (5)	6.060	2	0.98	4.7	2.13
		5	2.30		
		10	4.49		
		15	6.05		
0.080 (6)	12.500	2	0.50	2.25	4.44
		5	1.22		
		10	2.32		
		15	3.00		
0.039 (7)	25.64	2	0.26	1.2	8.33
		5	0.64		
		10	1.22		
		15	1.56		

[a]Number in parentheses refers to the respective curve in Fig. 57.

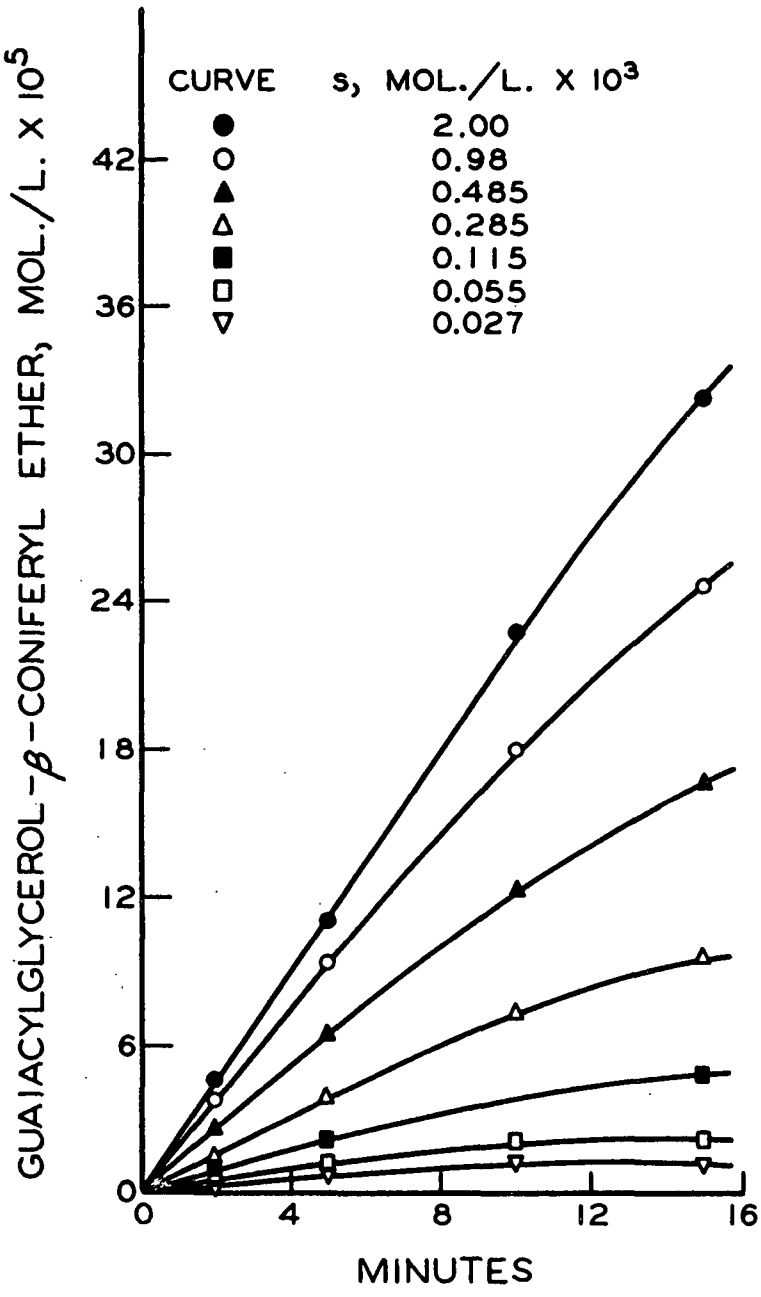


Figure 58. Moles Guaiacylglycerol-β-coniferyl Ether Occurring with Time in the Coniferyl Alcohol-Pseudolaccase K System in 0.05M Phosphate Buffer, pH 7.7

TABLE XLII

REACTION RATE DATA FOR THE OCCURRENCE OF GUAIACYLGLYCEROL-β-CONIFERYL ETHER IN THE CONIFERYL ALCOHOL-PSEUDOLACCASE K SYSTEM IN 0.05M PHOSPHATE BUFFER, pH 7.7, AT 25°C. AS OBTAINED BY RADIOCHEMISTRY

Substrate Concentration, $\underline{s}$, moles/liter x 10^3	$1/\underline{s}$, liters/mole x 10^{-3}	Time, minutes	Guaiacylglycerol-β-Coniferyl Ether, moles/liter x 10^5	Initial Rates Guaiacylglycerol-β-Coniferyl Ether, moles/liter-min. x 10^6	$1/\underline{v}$, guaiacylglycerol-β-coniferyl ether, liter-min$_5$/mole x 10^{-5}
2.00 (1)[a]	0.500	2	4.42	22.5	0.44
		5	11.00		
		10	22.80		
		15	32.40		
0.98 (2)	1.020	2	3.76	18.5	0.54
		5	9.30		
		10	17.90		
		15	24.80		
0.485 (3)	2.067	2	2.57	12.7	0.79
		5	6.41		
		10	12.30		
		15	16.60		
0.285 (4)	4.236	2	1.54	7.6	1.32
		5	3.90		
		10	7.40		
		15	9.50		
0.115 (5)	8.610	2	0.88	4.1	2.44
		5	2.20		
		10	--		
		15	4.85		
0.055 (6)	17.560	2	0.49	2.15	4.65
		5	1.22		
		10	2.20		
		15	2.30		
0.027 (7)	36.700	2	0.27	1.15	8.70
		5	0.66		
		10	1.24		
		15	1.14		

[a]Number in parentheses refers to the respective curve in Fig. 58.